THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. IX DECEMBER, 1928 No. 6

# FILTER AND CENTRIFUGE EXPERIMENTS WITH GUINEA-PIG VACCINIA VIRUS.

J. O. W. BLAND, L.M.S.S.A.,

Freedom Research Scholar.

*From the Hale and Dunn Clinical Laboratories, London Hospital.*

Received for publication October 10th, 1928.

THE question of the filtrability of vaccinia cannot be regarded as settled, since the recorded results of experiments with ordinary filter candles have been far from uniform. This difficulty in filtering vaccinia virus has been attributed to adsorption. Levaditi and Nicolau (1923) claim that vaccinia and other viruses pass through collodion membranes which retain completely or in major part hæmolysin, complement and diphtheria and tetanus antitoxin, and explain this somewhat anomalous finding on the ground that adsorption would be reduced to a minimum in the case of collodion ultra-filters. Bedson (1927) repeated Levaditi and Nicolau's experiments with herpes virus and failed to confirm them. In his hands herpes virus failed to pass through collodion membranes which allowed the passage of the least dispersed of the serum proteins in almost undiminished quantity, and he supported this finding by demonstrating that herpes virus could be concentrated by high-speed centrifugation. A possible explanation of this conflict of evidence may be sought in the fact that Bedson used a guinea-pig-skin strain of herpes virus and that Levaditi and Nicolau worked with a rabbit-brain strain. This view finds further support in the ultra-filtration studies of Zinsser and Tang (1927), who, working with a rabbit-brain strain of herpes, concluded that the virus particles were somewhere between 20 and 100$\mu$ in size. However, as Bedson pointed out, neither the experiments of Levaditi and Nicolau nor of Zinsser and Tang were suitably controlled, and in view of this it was decided to re-investigate the ultra-filtrability of vaccinia virus.

## MATERIALS AND TECHNIQUE.

*Virus.*—The strain of vaccinia (VcI) mainly used for this work was derived from calf-lymph, and propagated in guinea-pigs by scarification on the hairy skin. The strain has been described in a previous paper (Bland, 1928), where

also will be found the details of the preparation of tissue emulsions such as were employed for filtration tests together with the methods of titration of the virus. For the centrifuge experiments and for one filtration experiment a second strain was used (Vc X) which was also derived from calf-lymph, but which was propagated in the guinea-pig by intradermal injection in the pads. For experiment this strain was used in the form of a "phosphate diffusate" as described by Bedson (1927).

*Collodion membranes.*—Pyroxylin (British Drug Houses) was dissolved in various concentrations in a mixture of 25 parts of ether with 75 parts of absolute alcohol and to this solution 10% of glacial acetic acid was added. The collodion was poured on to a circular glass plate which was tilted in all directions so that the excess ran off and the whole plate was evenly covered. The membrane was left to evaporate for 5 minutes in the air, then plunged into water and separated from the plate when hardening had taken place. Discs of suitable size were cut from the membrane and were treated in one of two ways:

A. "*Untreated membranes*" were kept stored in tap-water or phosphate solution.

B. "*Treated membranes*" were soaked for 24 hours in a solution of 1/10 rabbit serum, washed in tap-water for 2–4 hours and stored in tap-water or phosphate solution.

*Filtration apparatus.*—The small Seitz filter was used as described by Bedson (1927). A negative pressure was used, varying in different experiments between 50 and 100 mm. of mercury.

*Diluent.*—The diluent fluid throughout was a sterile M/50 phosphate buffer solution of pH 7·6.

## FILTRATION EXPERIMENTS.

A general plan governed the whole series of experiments, which was developed under the following heads:

1. *The ease of filtration of the virus was to be compared with that of the guinea-pig serum proteins which take part in the serum precipitin reaction.*—These proteins are present in the material from which the virus suspensions are made to about 80–90% of those present in undiluted guinea-pig serum, so that virus suspensions could equally well be used as protein solutions and only one filtration was necessary through any one membrane.

The virus content of a suspension or filtrate was determined by titration, and thus the titre of the virus used in each experiment was known. This is important, since it is possible that the membrane itself may remove by adsorption some of the virus passing through it, which may only reach the filtrate in an amount less than 1 M.V.D. If the titre of the suspension is known it is possible to calculate the degree to which this diluting process may go without prejudicing the detection of the virus in the filtrate.

Two forms of the precipitin reaction were used to estimate the protein content. The first of these was the ordinary method in which the reaction is allowed to proceed to its end-point in a series of falling dilutions of antigen, the highest tube which shows a definite opalescence being regarded as the

titre. It was not possible to use this end-point because the virus suspensions themselves are turbid, and therefore the highest dilution in which definite particles were formed was adopted as the criterion. Since it may well happen in comparing any two solutions A and B that we find readings such as those given below (Table I) in which the end-points are not identical, no accurate comparison can be made between them by this method.

TABLE I.

| | Dilution of 1/10,000. | 1/20,000. | 1/50,000. |
|---|---|---|---|
| A | Heavy precipitate | Coarse particles | Turbidity. |
| B | Coarse particles | Fine particles | Turbidity. |

For more accurate comparisons recourse was had to a second method, which was based on the " optimal proportion " technique of Dean and Webb (1926). This method allows of a comparison of the antigen content of one solution with that of a second in percentage terms. The accuracy of this method depends solely on the narrowness of the steps between the falling dilutions of antigen.

2. *Each membrane was to be tested immediately after use for the presence of microscopic leaks.* This test was performed by filtering through the membrane, immediately after the virus suspension, 3 c.c. of a suspension of *B. paratyphosus B* containing 100 million bacilli per c.c. The filtrate of this was received direct into a tube containing 3 c.c. of a double-strength peptone water in which brilliant green was present in a dilution of 1/37,000. This tube was then incubated for 24 and 48 hours at 37°C., and at the end of each period loopfuls were sown on to plates of McConkey's medium. The plates were incubated at 37° C. for 24 hours, and any colourless colonies which developed were examined for their identity with *B. paratyphosus B* by their sugar fermentations and by their agglutinability in a high titre antiserum. This test was shown to be capable of detecting between 100 and 1000 organisms in the filtrate, and any membranes which it accused of passing *B. paratyphosus B* were held faulty and the experiments were condemned.

3. *Treated and untreated membranes were to be compared in respect of their permeability to virus and protein.* This subsidiary investigation was undertaken to confirm the results of Bedson (1927), who found that treatment of membranes with serum or cholesterol made them more permeable to protein. I also hoped that such treated membranes might prove more permeable to virus. In a few of the experiments the same membrane was used before and after treatment, but in the majority similar discs were cut from the same sheet of membrane, one of which was treated with serum while the other was left in water. They were compared together at the same time with the same sample of virus or protein solution and at the same filtration pressure.

*Results.*

The results of this series of experiments are displayed in Tables II and III, which speak for themselves. They amply confirm the observation of

Bedson that serum increases the permeability to protein, but, with one exception (Expt. 1, Table II), no membrane treated or untreated allowed the passage of vaccinia. This exception I believe to be explicable. In Table IV is given the result of the titration of virus and protein in this experiment, and this shows that the passage of virus is based on the appearance of a single vesicle, not in the lower but in the higher of the two dilutions of the filtrate. Furthermore this vesicle developed at least a day later than those on the control pig. This is therefore most probably an example of secondary infection brought about by the transference of infective material from one animal to another during examination. It occurred at a time when I had not realized the dangers of this and had not adopted the necessary preventive measures.

TABLE II.—*Experiments on Filtration of Virus through Treated and Untreated Membranes.*

| No. of experiment. | Filtrates from treated membranes. | | Filtrates from untreated membranes. | | Degree of dilution of virus allowed for in test. |
|---|---|---|---|---|---|
| | % Protein in or titre of filtrate. | Presence of virus. | % Protein in or titre of filtrate. | Presence of virus. | |
| 1 | 1/10,000 | 0 | > 1/1000 | + | 1000 fold. |
| 2 | 1/20,000 | 0 | > 1/1000 | 0 | 200 ,, |
| 3 | 60 % | 0 | > 15 % | 0 | 5 ,, |
| 4 | 75 % | 0 | > 15 % | 0 | 5 ,, |
| 5 | 80 % | 0 | > 20 % | 0 | 50 ,, |
| 6 | 80 % | 0 | > 5 % | 0 | 50 ,, |
| 7 | 44 % | 0 | — | — | 1000 ,, |
| | 57 % | 0 | — | — | |
| | 57 % | 0 | — | — | |
| 8 | — | — | 1/5000 | 0 | 2000 ,, |
| | — | — | 1/100 | 0 | |
| | — | — | 1/100 | 0 | 1000 ,, |
| | — | — | 1/100 | 0 | |
| 9 | > 1/1000 | 0 | > 1/1000 | 0 | 10 ,, |

In experiments 1, 2, 8 and 9 the precipitin test was by the end-point method. In the other experiments "optimal proportion" technique was used, and the protein content is expressed as a percentage of that present in the original suspension. Where the sign > is put before a % figure or fraction it indicates that this was the lowest limit employed in the test. The actual amount of protein passing these untreated membranes judging by the degree of particulation as compared with similar tests on known guinea-pig serum dilutions was probably less than 1%.

Experiment 9 was done on a diffusate of strain Vc X; such diffusates contain much less protein than the tissue emulsions of Vc I which were used for the other experiments.

TABLE III.—*Experiments on Filtration of Protein through Treated and Untreated Membranes.*

| No. of experiment. | Collodion. Strength. | Collodion. Batch. | Protein solution. | Treated membranes. Titre of, or % protein in, filtrate. | Untreated membranes. Titre of, or % protein in, filtrate. |
|---|---|---|---|---|---|
| 1 | 4% | 1 | Guinea-pig serum | 1/1000 | > 1/1000 |
| 2 | ,, | ,, | ,, ,, | > 1/1000 | > 1/1000 |
| 3 | ,, | ,, | ,, ,, | > 1/1000 | > 1/1000 |
| 4 | 2% | 2 | ,, ,, | None | Trace |
| 5 | 3% | ,, | ,, ,, | ,, | 10% |
| 6 | ,, | ,, | ,, ,, | About 1% | None |
| 7 | ,, | 1 | ,, ,, | 1/20,000 | > 1/1000 |
| 8 | ,, | ,, | ,, ,, | 1/20,000 | > 1/1000 |
| 9 | ,, | ,, | ,, ,, | 62·5% | > 3·1% |
| 10 | ,, | ,, | Virus | 1/10,000 | > 1/1000 |
| 11 | ,, | ,, | ,, | 1/20,000 | > 1/1000 |
| 12 | ,, | ,, | ,, | 60% | > 15% |
| 13 | ,, | ,, | ,, | 75% | > 15% |
| 14 | ,, | ,, | ,, | 80% | > 20% |
| 15 | ,, | ,, | ,, | 75–80% | > 10% |
| 16 | ,, | 3 | ,, | 80% | > 5% |

Experiments 1, 2, 3, 7 and 8 were done on the same membrane treated with serum between the first and second filtrations.

In experiments 4, 5 and 6 the pyroxylin was a bad batch which had been found unsatisfactory before and which gave a very opaque solution.

In experiments 1, 2, 3, 7, 8, 10 and 11 the precipitin test was by the end-point method. The titre of guinea-pig serum with the antiserum used was 1/100,000. The test was done by optimal proportions in the other experiments, and the protein content is expressed as a percentage of that present in the unfiltered solution.

TABLE IV.

| Days after inoculation. | Unfiltered virus. 1/100. | 1/1000. | 1/5000. | 1/10,000. | Filtrate. 1/100. | 1/1000. |
|---|---|---|---|---|---|---|
| 1 | ? | — | — | — | — | — |
| 2 | ? | ? | — | — | — | — |
| 3 | +++ | +++ | + | + | — | — |
| 5 | +++ | +++ | + | + | — | ∓ |

? = papules. +++ = confluent vesicles. + = discrete vesicles more than 6.
∓ = 1–3 vesicles. — = no take.

## CENTRIFUGATION EXPERIMENTS.

The filtration experiments suggested that the virus might be associated with particles large enough to be spun down by centrifugal force. Gordon (1925) believes this to be the case in rabbit vaccinia, and even thinks that the

virus will fall down under the action of gravity. Guinea-pig virus has not been examined from this point of view, and a short series of experiments was planned to investigate the matter.

For these experiments phosphate diffusates were chosen because they are more free from protein and contaminating bacteria than tissue emulsions. 5–10 c.c. of such a diffusate, or of a dilution of it, were placed in a sterile centrifuge tube and well mixed. A sample was withdrawn for titration and the rest centrifuged at high speed (about 6000 r.p.m.) for 2–2½ hours. The top 0·5 c.c. was carefully pipetted off and kept, the middle layers were discarded and the bottom 0·5 c.c. washed up and down on the deposit. These three samples, uncentrifuged virus, supernatant and deposit, were titrated, and care was taken that supernatant and deposit should be titrated on the same pig, to rule out any possible effect of varying susceptibility of the animals. An agar slope was also sown from each to gain a rough idea of the number of contaminating bacteria.

The results of the experiments are given in Table V. The reader's attention is called to the fact that in every case the deposits show a higher titre than the supernatants, and it follows that centrifugation has concentrated the virus in the lower layers of the fluid. This occurs independently of the degree of contamination by bacteria, and the result is therefore not due to the virus being carried down by them. That the deposits do not always show higher titres than the uncentrifuged virus can probably be explained on the grounds that different pigs were used for these titrations, and we here have coming into play the factor of varying susceptibility in the test animals.

## DISCUSSION.

The virus which has been used in this work is always retained by collodion membranes which allow the passage of the serum globulins, that is to say, the least dispersed of the serum proteins, and it is always to be found in greater concentration at the bottom than at the top of centrifuged samples. It is difficult to interpret this in any other sense than that this virus is associated with particles of considerable size. If it be objected that failure to pass the membranes is not due to size but to adsorption I would point out the work of Douglas and Smith (1928), who conclude that the particles of vaccinia carry a negative charge over a range of pH from 5·5 to 8·4. This observation considerably reduces the probability of the adsorbtion of vaccinia by filters which themselves carry a negative charge.

The experiments of course do not show whether these particles actually constitute the virus, or whether it is merely adsorbed to them as Bronfenbrenner (1927) believes to be the case with the bacteriophage. That it is not adsorbed to proteins is evident from the work of Douglas and Smith (1928), who were able to separate the virus from protein by cataphoresis on the acid side of pH = 6·8. It is of course just possible that the virus is adsorbed to other aggregates, and if this is the case it may account for the conflict between these results and those of Levaditi and Nicolau, since the particles with which the virus associates itself may not be so large in the case of neuro-vaccine stains as they are in pad and skin lesions. On the other hand Levaditi and Nicolau do not

TABLE V.—*Centrifuge Experiments.*

| No. of expt. | Uncentrifuged virus. Virus content. 1/1. | 1/10. | 1/100. | Bacterial content. | Virus centrifuged 2–2½ hours at 6000 R.P.M. Supernatant. Virus content. 1/1. | 1/10. | 1/100. | Bacterial content. | Deposit. Virus content. 1/1. | 1/10. | 1/100. | 1/1000. | Bacterial content. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | ± | ∓ | *Nil* | 0 | — | — | *Nil* | 0 | ++ | ∓ | — | *Nil.* |
| 2 | 0 | + | ∓ | ,, | 0 | — | — | ,, | 0 | ∓ | — | — | ,, |
| 3 | +++ | + | ... | 1 colony staphylococcus | — | — | — | ,, | +++ | ++ | ∓ | — | 3 colonies staphylococcus. |
| 4 | — | — | — | *Nil* | — | — | — | ,, | ∓ | — | — | — | *Nil.* |
| 5 | +++ | +++ | — | Semi-confluent growth of Gram-negative bacillus | + | ± | — | Discrete colonies Gram-negative bacillus | +++ | +++ | ∓ | ... | Confluent growth Gram-negative bacillus. |
| 6 | ++ | + | ∓ | *Nil* | ++ | + | — | *Nil* | +++ | ++ | + | ∓ | 7 colonies staphylococcus. |
| 7 | ++ | + | ∓ | ,, | 0 | — | — | ,, | 0 | + | + | — | 1 colony staphylococcus. |

+ + + = Confluent vesicles. + + = Semi-confluent vesicles. + = Discrete vesicles more than 6. ± = 3–6 vesicles. ∓ = 1–3 vesicles. — = No take. 0 = No titration at this dilution. Bacterial content = number of colonies developing on an agar slape from 1 large platinum loopful of material.

record any examination of their membranes for microscopic leaks, and their positive results may have been due to faulty membranes. A series of experiments with neuro-vaccine, similar to those recorded here, is now in progress. It is hoped that they will provide a final answer to this question.

### SUMMARY AND CONCLUSIONS.

1. Vaccinia virus from guinea-pig skin lesions fails to pass collodion membranes which allow the passage of serum globulins.

2. Confirmation is given to the observation of Bedson that treatment of such membranes with serum increases their permeability to protein.

3. Vaccinia virus from guinea-pig pad lesions can be thrown down by high-speed centrifugation for 2–2½ hours.

4. The particles with which this virus is associated are of considerable size and possibly within the limits of microscopic visibility.

---

The author desires to express his thanks to Dr. S. P. Bedson for the help and advice which he has so freely given throughout this work.

### REFERENCES.

BEDSON, S. P.—(1927) *Brit. J. Exp. Path.*, **8**, 470.
BLAND, J. O. W.—(1928) *Ibid.*, **9**, 97.
BRONFENBRENNER.—(1927) *J. Exp. Med.*, **45**, 873.
DEAN, H. R., AND WEBB, R. A.—(1926) *J. Path. Bact.*, **29**, 473.
DOUGLAS, S. R., AND SMITH, W.—(1928) *Brit. J. Exp. Path.*, **9**, 213.
LEVADITI, C., AND NICOLAU, S.—(1923) *C.R. Acad. Sci.*, Paris, **176**, 717.
ZINSSER, H., AND FEI FENG TANG.—(1927) *J. Exp. Med.*, **46**, 357.

---

## NATURAL AND ARTIFICIAL STIMULI IN THE PRODUCTION OF HUMAN DIPHTHERIA ANTITOXIN.

SHELDON F. DUDLEY.

*From the Royal Naval College, Greenwich.*

Received for publication October 9th, 1928.

IN 1922 the writer published a series of Schick test statistics from a residential school, which, during the two years previous to the time the inhabitants were Schick-tested, had suffered from two sharp outbreaks of diphtheria. When the boys of this school were divided into three groups according as to whether they had escaped both epidemics or passed through one, or two, the relative numbers of Schick-negative reactors in the groups

increased progressively from the sample which had least to that having most opportunity of contact with diphtheria cases. Therefore it was concluded that human circulating diphtheria antitoxin was stimulated by the diphtheria bacilli, or their products, in the environment.

This deduction has been confirmed in many ways, and it is usually accepted as proven that immunity to diphtheria can be acquired latently, without symptoms of illness, by subclinical or carrier infection, or even, perchance, by the mere inhalation and destruction of diphtheria bacilli or their products. Again, numerous series of Schick tests from different social groups and institutions have definitely shown that the higher the ratio of negative to positive reactors in a community, the easier it is to actively immunize the Schick-positive reactors in the community. This is what would be expected if it is true that a negative Schick reaction means previous exposure to diphtherial antigens. But so far as the writer knows it has not heretofore been possible to demonstrate the truth of this hypothesis directly on an adequate sample of human beings. Therefore the following attempt to do so may be of interest.

## NATURAL STIMULATION OF HUMAN DIPHTHERIA ANTITOXIN.

In January, 1928, it was decided to immunize a school of about 1000 boys, where diphtheria had been endemic for over three years. 906 boys were Schick-tested; the Schick technique was precisely the same as that previously described. The results are given in Fig. 1, which practically explains itself. The horizontal columns on the right-hand side of the diagram refer to different samples of boys. These groups each form a term entry, and are arranged according to seniority in the school. Thus the column marked 0 represents new entries who were Schick-tested before mingling with the rest of the school; while 10+ refers to boys who had been in residence 10 or more school terms. The left-hand series of columns is an index of the amount of diphtherial contact that the sample of boys, which is represented by the right-hand column immediately opposite, had experienced before being tested. The black ends of the left-hand columns are proportional to the number of cases of diphtheria that occurred during each of the periods indicated. The whole of each left-hand column is the sum of all the black pieces above it, and the figures 0–173 are the number of cases of diphtheria which were notified during the time that the corresponding sample of boys were in the school. The left-hand zero again refers to new entries who had had no chance of coming into contact with the school diphtheria bacilli before they were Schick-tested. However, these new entries, together with all the other boys, had had, on the average, 12 years' experience of the English diphtherial environment normal to their class before they joined the Greenwich Hospital School. The average percentage of Schick-positive reactors found in 6 batches of new boys who were tested at various periods between 1921 and 1928 was 53. Therefore the group of new entries used to make Fig. 1 was an exceptionally susceptible batch since only 32% were Schick-immune. In Fig. 1 the Schick-negative reactors amongst the new boys are represented by the diagonally shaded portion of the uppermost column, and this diagonal shading is carried straight down all the other columns level with the end of the new entries column in order to envisage the immunity

## NATURAL STIMULATION OF DIPHTHERIA ANTITOXIN.

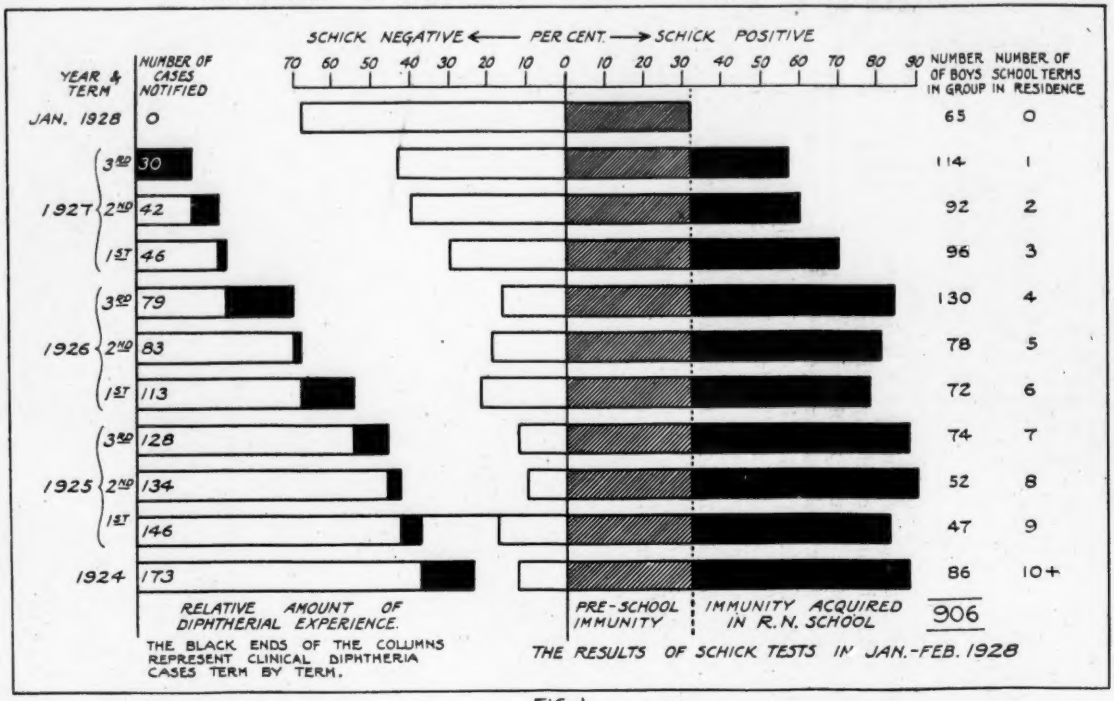

SCHICK NEGATIVE ← PER CENT. → SCHICK POSITIVE
70 60 50 40 30 20 10 0 10 20 30 40 50 60 70 80 90
YEAR & TERM
NUMBER OF CASES NOTIFIED
NUMBER OF BOYS IN GROUP
NUMBER OF SCHOOL TERMS IN RESIDENCE
JAN. 1928 0 65 0
1927 3RD 30 114 1
1927 2ND 42 92 2
1927 1ST 46 96 3
1926 3RD 79 130 4
1926 2ND 83 78 5
1926 1ST 113 72 6
1925 3RD 128 74 7
1925 2ND 134 52 8
1925 1ST 146 47 9
1924 173 86 10+
906
RELATIVE AMOUNT OF DIPHTHERIAL EXPERIENCE.
PRE-SCHOOL IMMUNITY
IMMUNITY ACQUIRED IN R.N. SCHOOL
THE BLACK ENDS OF THE COLUMNS REPRESENT CLINICAL DIPHTHERIA CASES TERM BY TERM.
THE RESULTS OF SCHICK TESTS IN JAN.-FEB. 1928

FIG. 1.

which was acquired by all boys before joining the school. In most groups the division between shaded and black would have been in reality nearer the 50% than the 30% position, but owing to the exceptionally low herd-immunity of this special batch of new entries and in order to avoid complicating the diagram the division was drawn at the 32% level. The black parts of the right-hand columns may be considered to represent the immunity that was acquired naturally during residence at school. This diagram shows that the decline of diphtheria susceptibility was gradual and continuous. This is because diphtheria was endemic in the school during the whole period concerned. During another period (see Dudley, 1922), when there were two distinct epidemics, with an almost complete absence of diphtheria cases between them, the herd-immunity of the boys when arranged according to their seniority in school increased in two abrupt steps. The correlation between herd-immunity and the amount of bacterial contact, which is obtained by testing different samples of varied experience at the same time, is really only circumstantial evidence that the excess of Schick-negative reactors in the senior samples acquired their immunity while in the school. More direct evidence has been obtained at Greenwich on many occasions by testing the same batches of boys at different times. For example 83 boys who were susceptible Schick-reactors in 1922 were re-tested in 1924, exactly two years later; 48 of these boys, or 58% of the sample, were found to have changed their Schick reaction from positive to negative in the interval between these two tests.

The immunity, which is naturally stimulated by a diphtherial environment, is not produced altogether without symptoms. During 6 years at Greenwich it was calculated that one boy became a clinical case of diphtheria while three were latently immunized. But owing to the special character of this environment this case-to-carrier ratio is higher than the average for London as a whole.

## ARTIFICIAL STIMULATION OF HUMAN DIPHTHERIA ANTITOXIN.

There were 243 positive Schick-reactors among the 906 boys that Fig. 1 refers to. These susceptibles were all inoculated with diphtheria prophylactics, but the following investigation is confined to 209 boys who were available at the right times to go through the same routine course of active immunization—that is to say, this group were all inoculated and Schick-tested at the same intervals of time. Two antigens were employed in this experiment—(*a*) toxoid, (*b*) toxoid-antitoxin floccules as described by Glenny and Pope (1927). Each group of boys to be immunized was evenly divided into two samples of similar seniority, and each half group were all given one of the two antigens. When further doses of prophylactic were given to the same boy, he always received the same antigen as he had had before. This procedure eliminated any chance that the relative variations in the response of the groups to active immunization were due to any difference in the antigenic qualities of the two prophylactic agents used. The 209 susceptible subjects of this investigation were all given an equal dose of one or other of the diphtheria antigens. They were then Schick-tested 3, 6, 12 and 24 weeks later, and further equivalent doses of prophylactic were given, at 4 and 8 weeks' interval after the first dose, to those subjects whose

Schick reactions remained positive at the various re-tests. The boys were divided into three groups: (*a*) New entries, (*b*) boys who had been at school less than a year, and (*c*) boys who had been more than a year in residence. Table I and Fig. 2 gives the results of the Schick tests on these groups.

The table shows that three months after the first inoculation of diphtheria prophylactic 41% of the new boys still had positive reactions, while only 13% of the most senior group of the original susceptible reactors retained a positive Schick test. In six months 82% of the most junior group had become immune, but by this time 97% of the boys who had been more than a year at school had acquired an active immunity. In Table I the first and last columns give the percentage of positive reactors, before and after the course of prophylactic inoculations, which existed in the whole group of boys, including all the original negative reactors, who of course had not been artificially immunized. The

TABLE I.

BEFORE ← ACTIVE IMMUNISATION → AFTER

| PER CENT. POSITIVE REACTORS IN ORIGINAL SAMPLE | SENIORITY OF GROUP IMMUNISED | NUMBER OF SCHICK POSITIVES IN GROUP | POSITIVE REACTORS REMAINING AFTER INNOCULATION | | | | | | | | PER CENT. POSITIVE REACTORS IN ORIGINAL SAMPLE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 3 WEEKS | | 6 WEEKS | | 12 WEEKS | | 24 WEEKS | | |
| | | | NUMBER | PER CENT. | NUMBER | PER CENT. | NUMBER | PER CENT. | NUMBER | PER CENT. | |
| 68 | NEW ENTRIES | 34 | 27 | 79 | 22 | 65 | 14 | 41 | 6 | 18 | 12 |
| 38 | UNDER A YEAR | 103 | 70 | 60 | 34 | 33 | 26 | 25 | 8 | 7·8 | 3·0 |
| 16 | OVER A YEAR | 72 | 38 | 53 | 16 | 22 | 9 | 13 | 2 | 2·8 | 0·4 |
| 27 | TOTAL | 209 | 135 | 65 | 72 | 34 | 49 | 33 | 16 | 7·7 | 2·1 |

RATE OF IMMUNISATION INCREASES WITH DIPHTHERIAL EXPERIENCE, OR HERD-IMMUNITY, OF THE GROUP.

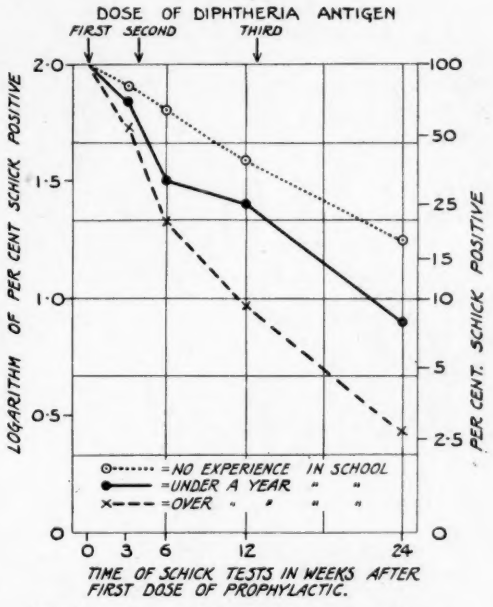
contrasts between these two sets of figures is a striking confirmation of the well-known observation, that the higher the proportion of immunes in a community the easier it will be to immunize the susceptibles in that community. In Fig. 2 are plotted the logarithms of the percentages of boys found immune, in the three seniority groups, at the time of each successive series of Schick tests. This method of presentation was used, since it makes the slope of the line between the points of reference proportional to the rate of immunization during the period between any two of the Schick tests series.

If Fig. 2 is examined carefully it is seen that owing to a happy chance (since the sample was so small) the points of reference for the decline in susceptibility of the new entries all fall in an almost straight line. This means the prophylactic doses had (also by chance) been so spaced that the rate of immunization remained constant throughout the whole period of 24 weeks. This rate was 30% of susceptibles per month to become immune. In the case of the two senior groups the rate in decline of susceptibility was not constant, as is indicated by the varying slope of their graphs. However, the most senior

boys became immune twice as rapidly as the most junior, since their average rate of immunization for the whole period was 60% per month. The intermediate group became immune at a rate of about 42% of residual susceptibles per month.

The slopes of the graphs in Fig. 2 show another point which may be of significance. The two senior groups became immune more rapidly during the first 6 weeks than during the latter part of the 24 weeks' period. This may indicate that these two groups contain more boys in a sensitized or "pre-immune" condition than the new entries did. In other words previous contact with *C. diphtheriæ* in the school environment had acted as a primary antigenic stimulus, which was followed by a more rapid response to a secondary stimulus in the shape of a mechanically injected diphtheria antigen. Thus this investigation closely parallels Glenny's (1925) experiments with diphtheria toxin and guinea-pigs, except that a dense diphtherial environment was the primary stimulus, and a herd of human boys were used in lieu of the guinea-pigs.

FIG. 2.




DECLINE IN SUSCEPTIBILITY IN ACCORDANCE WITH DIPHTHERIAL EXPERIENCE

## IMMUNITY IS EXPERIENCE, NOT AGE.

Table II is produced in case there may be anyone who still clings to the theory that latent immunization in diphtheria is a physiological function of age, and that the difference in the rapidity of response to the same immunizing antigen is wholly due to a variable genetic factor. Table II refers to the boys who had been more than one term at school; the new entries were not included in it.

The sample was first divided into boys who were over and under 14 years of age, and then each of these age-groups was subdivided according as the members had been at school for less, or more, than a year. Owing to the age of joining the school ranging from 11–14 it was possible for a boy of 13 to have been as long as two years at school, whereas a few boys of 14 may not have been as many months in residence. This overlap in age and seniority explains why the difference between the percentages of susceptible reactors among boys

of over a year and under a year's seniority is 22%, whereas the boys over 14, though *on the average* of greater seniority, are only 11% more immune than the group under 14. Therefore seniority, or experience of the school environment, was more closely correlated with a high degree of herd immunity than age *per se*.

But the most convincing argument that the degree of herd-immunity depends on experience of diphtheria bacilli rather than on age is obtained by contrasting the two sets of figures in Table II, which are surrounded by the thick lines, when it is at once seen that the younger but more experienced group contained relatively half as many susceptibles as the sample, which was less experienced, though older in years. Table II also shows that although the rapidity of response to an injected diphtheria antigen was not significantly different in the old and the young groups when undivided, yet 52% of the

TABLE II.

AGE AND SENIORITY IN SCHICK IMMUNITY.

| SENIORITY → | LESS THAN A YEAR | | | | MORE THAN A YEAR | | | | TOTAL RESIDENTS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AGE ↓ | NUMBER IN GROUP | PER CENT. SCHICK POSITIVE | NUMBER INNOCULATED | PER CENT. IMMUNE 3 WEEKS | NUMBER IN GROUP | PER CENT. SCHICK POSITIVE | NUMBER INNOCULATED | PER CENT. IMMUNE 3 WEEKS | NUMBER IN GROUP | PER CENT. SCHICK POSITIVE | NUMBER INNOCULATED | PER CENT. IMMUNE 3 WEEKS |
| UNDER 14 YEARS | 232 | 40 | 183 | 33 | 185 | 16 | 27 | 52 | 417 | 29 | 110 | 37 |
| OVER 14 YEARS | 70 | 33 | 20 | 32 | 354 | 15 | 45 | 44 | 424 | 18 | 65 | 40 |
| TOTALS | 302 | 38 | 103 | 32 | 539 | 16 | 72 | 47 | 821 | 24 | 175 | 38 |

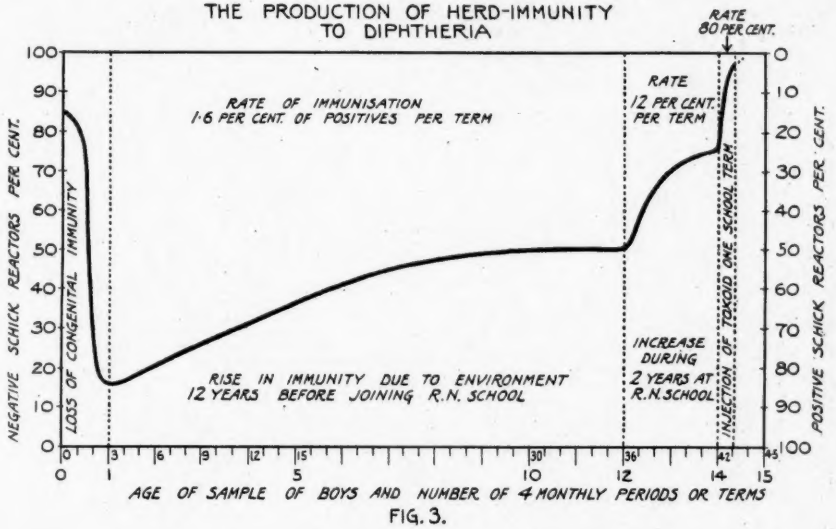
NOTE: CONTRAST FIGURES ENCLOSED IN THICK LINES.

young seniors became immune during the time that only 32% of the old juniors changed their Schick reactions from positive to negative. The statistics in Table II are strong evidence that the rate of response to diphtheria antigen is also principally determined by the amount of previous contact with *C. diphtheria* or its products. That differences in the genetic composition of an individual may be involved to some extent is not improbable. The 5 to 10% of Schick-positive reactors who resist all attempts to actively immunize them for a disproportionate time, indicate that there are inborn variations in the plasticity of the mechanism by which the organism adapts itself to the bacterial environment; but for all that, the degree of previous bacterial experience still remains the chief factor which determines the variation in the rate of response to antigenic stimuli.

## NATURAL *VERSUS* ARTIFICIAL STIMULATION OF HUMAN DIPHTHERIA ANTITOXIN.

Fig. 3 envisages an ideal curve of the production of herd-immunity in a hypothetical group of children who are supposed eventually to join Greenwich

Hospital School. This group is born together, 85% immune. During the first year of life most of this maternal immunity vanishes, so that at the age of 1 year the herd is only 15% immune. For the next 11 years herd-immunity is slowly acquired, in response to the environmental stimuli which are normal to the social class of the groups, and by the age of 12, when the herd enters Greenwich School, 50% of its members are Schick-negative reactors. The imaginary group now enters the school environment, where the density of diphtheria bacilli is much greater than they have been previously


THE PRODUCTION OF HERD-IMMUNITY TO DIPHTHERIA
RATE 80 PER CENT.
RATE OF IMMUNISATION 1·6 PER CENT. OF POSITIVES PER TERM
RATE 12 PER CENT. PER TERM
LOSS OF CONGENITAL IMMUNITY
RISE IN IMMUNITY DUE TO ENVIRONMENT 12 YEARS BEFORE JOINING R.N. SCHOOL
INCREASE DURING 2 YEARS AT R.N. SCHOOL
INJECTION OF TOXOID ONE SCHOOL TERM
NEGATIVE SCHICK REACTORS PER CENT.
POSITIVE SCHICK REACTORS PER CENT.
AGE OF SAMPLE OF BOYS AND NUMBER OF 4 MONTHLY PERIODS OR TERMS
FIG. 3.

accustomed to, hence in the course of the next two years the percentage of immunes in the herd increases to 75. The remaining susceptibles are now mechanically stimulated with a diphtheria antigen and within four months the herd becomes 95% immune. The rates at which the susceptibility of this hypothetical herd decline may be found by substituting the above times and numbers in the expression of the compound interest law,

$$r = \frac{100}{t} \log_e \frac{Q_o}{Q_t},$$

where the rate of immunization $r$ = the percentage of the residual Schick-positive reactions, present at any instant of time during the period $t$, which become immune during the next unit of time. $Q_o$ and $Q_t$ are the percentages of positive reactions which are present at the beginning and end of the period $t$, during which the immunizing stimulus is at work. $t$ is measured in school terms (*i. e.* 4 monthly units). Hence the rates of immunization in the example given were 1·6 between the ages of 1 and 12 in the average English environment,

12% per term in Greenwich Hospital School until the rate was increased to 80% of susceptibles to become immune per term by the artificial stimulus of injected diphtheria prophylactics. If this rate of immunization is used as an index of the intensity of the antigenic stimulus, then injections of laboratory-made prophylactics were 50 times as powerful as the natural immunizing influences of the average English environment, and in the same time the injected prophylactic was able to produce seven-fold the effect of a specific environment, in which diphtheria had been endemic for some years. Also natural auto-vaccination by the bacterial environment, besides being much slower than artificial methods, is of necessity accompanied by a heavy toll in clinical disease.

## CONCLUSIONS.

1. Circulating human diphtheria antitoxin is due to contact with toxigenic *C. diphtheriæ* or its products, which may be either in the natural environment, or hypodermic syringes.

2. The percentage of negative Schick-reactors in any group is a function of the amount of previous contact with *C. diphtheriæ*.

3. The rate at which the positives from any environment can be immunized is a function of the ratio between immunes and susceptibles in that environment. Therefore the rapidity of response to immunising injections of diphtherial antigens also depends on the amount and nature of previous contact with *C. diphtheriæ*.

4. Age *per se*, and differences in the genetic make-up of an individual, are much less important factors than previous bacterial experience in determining the degree of immunity which is possessed by an individual or a herd.

5. The production of human circulating antitoxin by the injection of manufactured diphtherial antigens causes a far more rapid increase in herd-immunity than can be produced by any immunizing stimuli which may exist in the natural human environment.

---

I must again thank Surgeon-Captain P. May, R.N., the School Medical Officer, for his trouble and assistance in arranging the administration details for this work, and my colleague Surgeon-Commander H. B. Parker, R.N., who assisted me with the Schick and prophylactic inoculations. Surgeon-Captain May and I are also deeply indebted to Dr. R. A. O'Brien for valuable advice and supplying us with the diphtheria prophylactics which were used for this work. This investigation was also designed to test the relative efficiency of the two antigens mentioned, but this part of the work is still incomplete, which explains why nothing more was said about the diphtheria prophylactics used than was necessary for the present thesis.

## REFERENCES.

DUDLEY, S. F.—(1922) *Brit. J. Exp. Path.*, **3**, 204.
GLENNY, A. T.—(1925) *J. Hyg.*, **24**, 301.
*Idem*, AND POPE, C. G.—(1927) *J. Path. Bact.*, **30**, 587.

# A COMPARISON BETWEEN THE ANTIGENIC PROPERTIES OF WHOLE AND LIPOID-FREE SERA.

SYBIL H. C. ROBINSON.

*From the Hale Clinical Laboratory, London Hospital.*

Received for publication October 10th, 1928.

IT is doubtful whether lipoids in a pure state are antigenic, but there is evidence that in combination with protein they are capable of giving rise to antibodies which react specifically with pure lipoid (Landsteiner and Simms, 1923; Sacks and Klopstock, 1925). In the case of the Forssman antigen the specificity would appear to depend on the lipoid radical attached to the protein and the investigation of Landsteiner and van der Scheer (1925, 1926) indicate that the same holds true in the case of anti-cell sera such as a hæmolysin. Hartley (1925) has demonstrated the importance of lipoid in the precipitin reaction, for he found that the removal of the ether soluble substance from both antigen and antibody prevented the reaction from taking place, whereas if only one of the two reagents were lipoid-free precipitation occurred. Scimone and Torii (1923) found that the use of a lipoid-free serum as an antigen led to the production of antisera which were more specific, but little has been done on this point. The following experiments have been carried out with lipoid-free horse and ox sera kindly placed at my disposal by Dr. L. F. Hewitt, in order to determine whether they differed from unextracted sera in antigenic property.

The horse and ox sera were freed from lipoid by Hewitt's modification of the method of Hardy and Gardiner (1927). Antisera were prepared in rabbits to the extracted and unextracted sera by giving four or five intravenous inoculations, 3·0 c.c. quantities at intervals of four days. The precipitin tests were carried out by mixing one part of the undiluted antiserum with nineteen parts of progressive dilutions of antigen. Readings were made after 2 hours' incubations at 37° C., and then after standing overnight in the ice-chest. The final results are summarized in Table I:

TABLE I.

| Anti-serum (1 drop), prepared from— | Antigen (19 drops). | 1/10. | 1/100. | 1/1000. | 1/10,000. | 1/100,000. | 1/200,000. |
|---|---|---|---|---|---|---|---|
| Whole horse serum | Horse | ++ | ++ | + | ∓ | − | − |
| | Extr. horse | ∓ | + | + | ∓ | − | − |
| | Ox | − | ∓ | ∓ | − | − | − |
| | Extr. ox | − | ∓ | − | − | − | − |
| Extracted horse serum | Horse | ∓ | + | ++ | ± | ∓ | ∓ |
| | Extr. horse | − | + | ++ | ± | ± | ∓ |
| | Ox | − | − | − | − | − | − |
| | Extr. ox | − | − | − | − | − | − |
| Whole ox serum | Horse | − | ∓ | ∓ | ∓ | − | − |
| | Extr. horse | − | ∓ | ∓ | − | − | − |
| | Ox | ± | + | + | ∓ | ∓ | − |
| | Extr. ox | + | + | ∓ | ∓ | − | − |
| Extracted ox serum | Horse | − | − | − | − | − | − |
| | Extr. horse | − | − | − | − | − | − |
| | Ox | − | ∓ | ± | ∓ | − | − |
| | Extr. ox | ∓ | ∓ | ∓ | ∓ | − | − |

++, +, ±, ∓ = different degrees of precipitation; − = none.

These findings confirm the observation of Scimone and Torii that the use of a lipoid-free serum as an antigen gives an antiserum which differs from one prepared with an unextracted serum in being more specific in its action.

---

My thanks are due to Dr. S. P. Bedson for his interest and help and to Dr. L. F. Hewitt who kindly placed the lipoid-free sera at my disposal.

## REFERENCES.

HARTLEY, P.—(1925) *Brit. J. Exper. Pathol.*, **6**, 180.
HEWITT, L. P.—(1925) *Biochem. J.*, **21**, 216.
LANDSTEINER, K., AND SIMMS, S.—(1923) *J. Exper. Med.*, **38**, 127.
LANDSTEINER, K., AND VAN DER SCHEER, J.—(1925) *Ibid.*, **41**, 427; (1926) *Ibid.*, **42**, 123.
SACKS, H., AND KLOPSTOCK, A.—(1925) *Biochem. Zeitschr.*, **159**, 491.
SCIMONE AND TORII.—(1923) *Ztschr. f. Immunitätsforsch.*, **38**, 264.

---

# A STUDY OF THE BACTERICIDAL POWER OF "WHOLE" BLOOD BY THE METHOD OF CULTURE IN SLIDE-CELLS.

J. M. ALSTON.

*From the Bacteriology Department, University of Edinburgh.*

Received for publication June 21st, 1928.

IN 1923, Wright, Colebrook and Storer introduced a method by which the bactericidal power of whole blood may be estimated by implanting and culturing bacteria in defibrinated blood. This procedure presents the apparent advantage that it tests the bactericidal action of both the serum and the phagocytic corpuscles while they remain in their natural relationship to one another. The method, which is fully described and illustrated in the original paper, was first used by Wright and his co-workers to study changes in the bactericidal power of the blood during the course of an infection, and to guide the application of immunological therapy and indicate its effects. Several workers have subsequently extended the use of the method. Colebrook, Eidenow and Hill (1924) found that in man and in certain animals an increase in the bactericidal power of the whole blood and of the serum occurred after exposure to various radiations which produced an inflammatory reaction of the skin. Maitland (1926) showed that after subcutaneous injections of staphylococcus vaccines the bactericidal power of the blood of normal persons

was increased for the homologous organism. Colebrook and Storer (1924) reported that the addition to freshly drawn blood of the minimum amount of a decalcifying agent necessary to prevent clotting, *e.g.* 0·25–0·5% of sodium citrate, considerably reduced its power to kill staphylococci and streptococci, but that if the blood had been defibrinated before the decalcifying solution was added there was only a comparatively slight reduction. They concluded also from their experiments that the decalcifying agent produces its effect by interference with the functions of the leucocytes and not by lowering the opsonic power of the serum. The results of Walsh and Harmsworth (1926) led them to agree with the last-mentioned workers that defibrinated blood has more bactericidal power than blood in which clotting has been prevented by the addition of 0·25% of sodium citrate, but they found, in contradiction to Colebrook and Storer, that the addition of sodium citrate solution to defibrinated blood inhibited the growth of implanted staphylococci. Fleming (1926) noted that the bactericidal power of defibrinated whole blood was diminished when, *in vitro*, its salt concentration was either decreased beyond a certain point by the addition of sterile water, or increased to a certain degree by the addition of hypertonic sodium chloride solution. He stated also that the intravenous injection in rabbits of hypertonic solutions of sodium chloride caused an immediate decrease of the bactericidal power of their blood followed by an increase to a value higher than that before injection.

In the experiments which form the subject of this paper, a study has been made of the bactericidal power of the whole blood by the application of the method of Wright, Colebrook and Storer, with particular reference to (1) the technique and its limitations and the reliability of the method, especially for comparative tests; (2) the significance of comparisons of the bactericidal power of specimens of blood withdrawn at different times from normal persons and animals, and of comparisons between the bactericidal power of specimens drawn before and after the application of certain agents supposed to influence antibacterial resistance. Some preliminary attempt has also been made to analyse the mechanism of bactericidal action by whole blood.

## TECHNIQUE.

The technique followed was in accordance with that originally described by Wright, Colebrook and Storer, and the general method need not be detailed here. The question of the optimum dilution of the suspension used was found to be specially important and will be referred to more fully later.

It was found that familiarity with the technique brought a regularity of method which diminished variations in the results due to *experimental error*, but such variations, although slight, cannot be altogether ignored. The results of twelve simultaneous tests on one specimen of blood are given to show the degree of consistency which can be reached in using the test. In these tests the inoculum was a relatively heavy one and the numbers of colonies were correspondingly large. Counting was effected by careful ruling of the slide into sections. Numbers of colonies: 293, 271, 264, 280, 285, 270, 262, 279, 258, 284, 265, 274; or in ascending order—258, 262, 264, 265, 270, 271, 274, 279, 280, 284, 285, 293.

The figures range from 258 to 293, and are fairly evenly distributed within that range, the average, 274, being nearly equal to the mean between the two extremes, 275. The highest figure is 13·5% greater than the lowest.

Since the experimental error is reduced when colonies are numerous as compared with tests in which there are only a few, some series of results in which the number of developing colonies was smaller than in that quoted above will be given. The results of four simultaneous and identical tests with one specimen of blood were 67, 69, 70, 75; average 70. The colony counts of another similar series were 34, 35, 37, 28; average 33·5.

These results show that even after considerable practice in the method there is a certain experimental error, and because of this nearly all tests were carried out four-fold; the figures, of which an average was taken, were made use of only when they were reasonably consistent. It was found to be especially important that the *bacterial emulsion* used should be as far as possible a suspension of single organisms without clumps, and to ensure this it was shaken thoroughly with glass beads after large visible clumps had been removed by centrifuging.

The question of ascertaining the *number of viable organisms originally mixed with the blood or serum* was considered so that the number of colonies resulting after incubation might be expressed as a percentage of the number of organisms implanted. If the number of organisms is estimated by any method of direct enumeration apart from plating, no information is gained as to what proportion are viable. On the other hand, when an enumeration is carried out by plate cultures in the usual way, no medium has exactly the same qualities for growth as defibrinated blood, which is specially rich in growth-promoting substances, and contains as well varying amounts of anti-bacterial substances. For example, when 2·5 c.mm. of a dilute emulsion of staphylococcus were plated in ordinary nutrient agar, it was found that about an equal number of colonies resulted as in a slide-cell which contained the same amount of bacterial suspension and 50 c.mm. of blood. It may be assumed that a certain proportion of the viable organisms had been eliminated by the bactericidal agencies of the blood, so that apparently the richer medium supplied by the blood, as compared with ordinary nutrient agar, counterbalanced to some extent its bactericidal action.

No results have been recorded as percentages of the number of organisms inoculated, owing to the difficulty of determining accurately how many viable organisms are present in each inoculum used. Some workers, including Colebrook, Eidinow and Hill, Maitland, and Fleming, have recorded their results in terms of the percentage of viable bacteria killed, taking colony counts of the inoculum on agar plates as the criteria of viability, but although results appear more expressive when stated in this way, the refinement is in danger of being gained at the expense of accuracy.

In practice, and as a result of experience with the method, an endeavour was usually made to bring the number of colonies within the range of 20–100 by varying the density of the emulsion, according to the organism used and the expected bactericidal power of the blood or serum to be tested.

In the tests reported here the inoculum was usually prepared from 18 hours' broth culture in which the total number of organisms present (viable

and non-viable) was estimated by comparison with standard opacity tubes. The culture was then diluted with sterile physiological saline to such a degree that the amount of inoculum used in the test, namely, 2·5 c.mm., would contain 200–400 organisms in the case of staphylococci, and more in the case of *B. coli*. The dilution necessary for this purpose was between 1 in 2000 and 1 in 10,000 according to the amount of growth in the culture. The range of 200–400 organisms was chosen because it was found by experience that this produced with most specimens of blood a number of colonies which was neither too large for accurate counting nor too small for reliable comparisons.

It was found convenient for most purposes to use for each specimen of blood or serum only one concentration of inoculum, and to put up four tests, rather than use varying concentration and fewer tests with each. It was found also that the comparative bactericidal effect of the serum alone could be tested in slide-cells by the same method, discrete colonies developing in the serum in the same way as in blood, and an attempt was made to obtain some information regarding the factors concerned in the bactericidal action of whole blood by carrying out parallel tests with serum alone. When serum was tested, the emulsion used was half the density of that for whole blood, on account of the less bactericidal power of the serum *per se* and the greater number of colonies that developed.

The completeness or incompleteness of *defibrination* and the inclusion of corpuscles in the coagulum appeared to be factors that might influence results. In all the experiments recorded in this paper, blood was obtained from a vein either by means of a syringe or by venesection, and it was collected in sterile 25 c.c. centrifuge tubes, with round ends, which contained 8–12 sterile glass beads. After complete defibrination had been obtained by shaking for a few minutes the corpuscles and sera could be pipetted off without any clots, and there remained only a whitish mass of fibrin which, when examined microscopically, was seen to entangle only a few corpuscles.

To ascertain, in the first place, the degree of constancy of the bactericidal effect of different specimens withdrawn and defibrinated separately, specimens were taken one immediately after the other from the same animal, carefully defibrinated by the method described and then compared in the following experiments:

*Experiment.*—From a rabbit one specimen of blood was obtained by cardiac puncture with a syringe, and two specimens from an ear vein by venesection. All three were defibrinated as described: *Staphylococcus aureus* was the organism used for inoculation.

The number of colonies after incubation of the slide-cells were:

Heart blood, 36, 37, 32, 36; average, 35.
Ear vein blood (1), 39, 35, 36; average, 37.
„ „ (2), 42, 40, 36, 30; average, 39.

These results correspond closely within the limits of experimental error, and their constancy shows that any slight variation in manipulation of the three specimens during defibrination produced no relative change in their bactericidal power.

Corresponding results were obtained with sera separated by coagulation from specimens of blood obtained within a few minutes of one another from

the ear of a rabbit. The serum was substituted for whole blood in the slide-cell test.

The possibility that varying degrees of shaking of the blood with beads might produce artificial variations in the bactericidal power of the blood or serum by lysis of corpuscles was excluded by the results of the next experiment.

*Experiment.*—Two specimens of rabbits' blood were taken by venesection, and one was completely defibrinated by shaking for 4 or 5 minutes, while the other was shaken vigorously by hand for ¼ hour, and in a shaking-machine for 2 hours. *Staphylococcus aureus* was the organism used.

The numbers of colonies in the slide-cells with the whole blood were :

Blood, 5 minutes' shaking, 120, 130, 125, 142; average, 129.

„ 2¼ hours' shaking, 140, 128, 120, 135; average, 131.

The corresponding sera tested in place of the whole-blood specimens also produced approximately equal numbers of colonies.

A further question is the reliability of comparisons between specimens of blood kept for varying times after withdrawal. Thus, to compare accurately different specimens, it is obviously essential that they should be tested simultaneously with the same bacterial emulsion. This question is of particular importance when the method is employed for instituting comparisons in the bactericidal power of the blood before and after subjecting persons or animals to certain influences. Thus, specimens may have to be kept for several hours or longer before they can be tested along with the last specimen to be withdrawn.

To throw light on this question the following experiments were carried out :

*Experiment.*—Blood was withdrawn from a healthy rabbit at different times before the tests were performed, and all the specimens, except the one taken 10 minutes before the test, were kept in the ice-chest at 5°C., from the time of withdrawal until the test was carried out.

*Staphylococcus aureus* was the organism used, and the implantation in the serum was half of the blood.

| Interval between withdrawal and testing. | Colony count—whole blood (average of 4 tests). | Colony count—serum (average of 4 tests). |
|---|---|---|
| 10 minutes | 25 | 8·5 |
| 6 hours | 16 | 10·5 |
| 24 „ | 12 | 14 |
| 52 „ | 14 | 13 |
| 16 days | 20 | 14·5 |

Of the whole-blood specimens, the one tested almost immediately after withdrawal showed distinctly less bactericidal power than the other four; in the case of the serum the difference in the figures was smaller. It would appear remarkable that the bactericidal power of the whole blood was apparently greater after the lapse of 6 hours to 16 days than immediately after withdrawal, but in the interpretation of these results the possibility of a normal variation, *in vivo*, of the bactericidal power of the blood has to be

considered and experiments illustrating such variation will be recorded later. It is noteworthy, however, that the colony count in the case of a specimen kept for even 16 days is relatively comparable with that for a recently withdrawn specimen. Another experiment gave similar results.

*Experiment.*—Similar to the last two.

| Interval between withdrawal and testing. | Colony count—whole blood. | Colony count—serum. Implantation of half that of whole blood. |
|---|---|---|
| 10 minutes | 33 | ... |
| 6 hours | 33 | 17 |
| 24 „ | ... | 23 |
| 72 „ | 34·5 | 24 |
| 96 „ | 36 | 21 |

In this series the results in the case of whole blood were very consistent.

The question may now be discussed whether keeping blood *in vitro* has any artificial effect on its bactericidal power. This question is obviously important since the test is utilized for comparison of specimens kept for different periods. There is first the possibility that defibrinated blood kept *in vitro* might undergo a decline in bactericidal power. It is evident from the illustrative examples that there is no rapid decline in bactericidal power. Thus, the effect after 96 hours may be practically equal to that after 6 hours, and comparisons made of specimens kept for periods ranging from a few hours to 4 days may therefore be accepted as reliable. It will be noticed that the variations found in the series of results under review were mostly in the direction of greater bactericidal power in older as compared with more recent specimens. The question of a normal variation, *in vivo*, however, has to be considered, and it will be shown later that the variations seen in the present series may be due to this factor.

At present no comments will be made on the results of tests with serum, as they will be considered later in connection with the analysis of the bactericidal power of whole blood.

## DISCUSSION AND CONCLUSIONS REGARDING THE METHOD.

In assessing the practical applications of a method such as this, experiments indicating the degree of consistency that can be expected from results, and the influence of variations in technique, are of considerable interest.

The method was devised for tests with defibrinated whole blood, but it has been found an advantage, for purposes of comparison and of analysis of effects, to use it also for bactericidal tests with serum alone. Thus simultaneous tests with whole blood and with serum can be readily carried out by the same technique.

It is seen from the illustrative examples of tests performed that equal results are obtained with specimens of blood collected one immediately after the other and defibrinated in separate tubes. Also, so long as defibrination is complete, the length of time the specimens are shaken with beads makes

almost no difference, and there appears to be no deterioration of bactericidal power when blood is kept *in vitro* in the ice-chest for periods up to 4 days.

For comparative work there are two factors which introduce difficulty and uncertainty. One is that readings from each specimen of whole blood or serum are not absolutely uniform, but only relatively so, with the result that differences of less than 15% cannot be considered as significant.

The other difficulty is peculiar to tests with whole blood, viz. the number of organisms viable in a medium of serum and corpuscles without bactericidal power is not readily ascertainable. If this could be accurately estimated the number of killed organisms could be expressed as a percentage of the number implanted in order to indicate the absolute bactericidal power of the blood; to attempt this, however, as some workers have done, on the basis of a fallacious estimate of viable organisms inoculated, only hides and does not eliminate a disadvantage of the method.

## COMPARATIVE RESULTS OBTAINED.

### *Diurnal Variations in the Bactericidal Power of Human Blood.*

The original object of the investigation was to apply the slide-cell method for testing the effect, on the bactericidal power of the blood, of a number of agents supposed to produce some non-specific increase in anti-bacterial resistance.

In order to ensure a sound basis for comparison, tests were made of the bactericidal power of specimens of blood withdrawn from normal persons and animals at different times. The results of these tests showed that in both man and animals the bactericidal power of the blood may vary during the course of the day, and in the range of results obtained the variations were greater and more frequent in the human subject than in the case of laboratory animals.

*Experiment.*—Two specimens of blood were withdrawn by vein puncture from a healthy man, J. A—, one at 9 a.m., immediately before breakfast, and the other at 2.15 p.m., one hour after the mid-day meal.

The organism used was *Staphylococcus aureus* and the results were as follows:

| | 1st test. | | 2nd test. | | 3rd test. | |
|---|---|---|---|---|---|---|
| Inoculum . . . | *x | $\frac{x}{2}$ | x | $\frac{x}{2}$ | x | $\frac{x}{2}$ |
| Whole blood, 9 a.m. . | †6·5 | 2 | 8·5 | 1 | 55 | 40·5 |
| „ „ 3 p.m. . | 0 | 0·5 | 2·5 | 0 | 28·5 | 26 |

* Bacterial emulsions of different densities were used in each test, the number of organisms in the first (x) being double that in the second $\left(\frac{x}{2}\right)$.

† Each figure is the average of the number of colonies in two slide-cells.

The first test was carried out on the day after the withdrawal of the blood, and the test was repeated on each of the two succeeding days as a check on the first results, and to discover whether the increase of bactericidal power

found in the second specimen was maintained when the specimens were kept for three days *in vitro*.

These results show that the bactericidal power of the blood was markedly greater at 2.15 p.m. than at 9 a.m.

In several of the following experiments leucocyte counts were carried out, and the question of the leucocyte content as a factor in the variation of the bactericidal power will be discussed later.

Two experiments were carried out with specimens of the same person's blood and the same organisms. In one case there was a marked increase in the bactericidal power of the second specimen compared with the first, and in the other case there was not a significant difference. The leucocyte counts in the two cases showed no appreciable change.

*Experiment.*—Similar experiment with the blood of another person, J. M. A—:

| | | Leucocyte count. |
|---|---|---|
| Whole blood, 9 a.m. . . . . | *145 | 5,600 |
| „ „ 3 p.m. . . . . | 101 | 6,000 |

* Each figure is the average of four tests.

The result shows an apparent increase in the bactericidal power of the second specimen, but no significant change in the leucocyte count.

A similar comparative test with specimens of blood taken just before a meal at mid-day and one hour after respectively elicited no appreciable variation.

The results of these experiments show striking diurnal variations in the bactericidal properties of the blood. The question of their relation to the ingestion of food and the "digestive" leucocytosis will be discussed later.

In the previous experiments it has been shown that when specimens of blood are kept for different periods *in vitro* at a low temperature their bactericidal power does not deteriorate rapidly, nor in such a way as to seriously invalidate the results of simultaneous comparative tests carried out with them. When specimens were withdrawn from the same animal at different times, kept under the same conditions and tested together, the degree of bactericidal power revealed had no uniform relationship to the age of the specimen; in some cases specimens differing in age from a few minutes to several days showed almost equal bactericidal power; in other cases the older specimens showed more bactericidal power than the more recently withdrawn blood, and *vice versâ*. To account for such results, it has been suggested that variation in bactericidal properties may occur *in vivo*, and this is borne out by the results of the experiments with specimens of human blood drawn at different times of the day. In this particular series of tests the possible relative changes brought about by keeping the specimens can be further discounted owing to the short time intervening between the taking of the specimens. Further, the bloods were kept till the following day before testing.

It may be concluded, therefore, that in the human subject the bactericidal power of the blood is not constant throughout the day. Also, in view of the fact that the first specimen, withdrawn before the morning or mid-day meal,

was generally less actively bactericidal than the second, which was taken in the afternoon, it might seem reasonable to suppose that the ingestion of food may have the effect of producing a temporary rise in the bactericidal power of the blood. This suggestion is made on an analogy with the commonly accepted idea of "digestive" leucocytosis, although in the tests recorded the variations in the leucocyte counts, and the corresponding specimens of blood for leucocyte count and for determination of bactericidal power were withdrawn in all cases within 2–5 minutes of each other. But the work of Shaw (1927), published since the results under consideration were obtained, provides evidence that the normal diurnal variations in the numbers of leucocytes in man occurs in two daily tides which ebb and flow independently of sleep, ingestion of food or exercise, and that the highest counts are obtained in the afternoon and during the early hours of the morning. The experiments recorded above are not extensive enough to show whether or not the fluctuation of bactericidal power of the blood is of a similar nature.

*Diurnal Variation in the Bactericidal Power of Rabbit's Blood.*

A series of experiments was carried out to ascertain whether there is any diurnal variation in the bactericidal power of the blood of normal rabbits—the animal selected for experimental tests in this investigation. The animals were allowed normal access to their food in view of the possibility that deprivation of food might produce a disturbance of the bactericidal power. *Staphylococcus aureus* was the organism used in each case.

The figures in the tables are averages of four tests.

*Experiment.*—Blood was withdrawn by venesection from a healthy rabbit at three different times in the same day. The specimens were tested twice—on the day of withdrawal and on the next day after being kept in a refrigerator at 5° C. overnight.

| Time of withdrawal of specimen. | *1st test.* Average colony count. | *2nd test.* Average colony count. | Leucocyte count. |
|---|---|---|---|
| 11.30 a.m. | 44·5 | 46 | 5,450 |
| 3 p.m. | 46 | 40 | 6,300 |
| 5 p.m. | 42·5 | 40·5 | 5,700 |

In this experiment the three specimens exhibited no appreciable difference in bactericidal power.

In five further experiments of a similar nature variations of greater or less degree were noted, but in only one was the variation considerably beyond the limit of experimental error. In this case the leucocyte count showed an increase parallel with that of the bactericidal power, but in the other four experiments the changes in the number of white corpuscles were relatively small.

These experiments on rabbits indicate that the bactericidal power of their blood is normally more constant than in the human subject, although the last experiment quoted shows that the constancy is not absolute.

A possible source of error in work of this sort, where comparisons are made between different specimens of blood, is that the withdrawal of blood in certain quantities might itself produce an alteration in the bactericidal and other immune properties of the blood remaining in the circulation, which might fallaciously be attributed to the action of some agent used for experimental or therapeutic purpose. In view of this, the minimum amount of blood was withdrawn in all cases and the continuance of bleeding after sufficient had been obtained was prevented. From the human subject 3 to 5 c.c. were usually taken, and from rabbits 1, 2, or sometimes 3 c.c.

## OBSERVATIONS ON THE EFFECTS OF VARIOUS AGENTS.
## ULTRA-VIOLET RADIATION.

### (A) *Children Exposed for the First Time to the Mercury Vapour Lamp for Short Periods.*

These observations were made on four children from 1 to 4 years. The time of exposure (of the whole body) to radiation was 4 minutes or 5 minutes, and blood was taken immediately before treatment and 5 or 15 minutes after it.

For the bactericidal tests the organism used was *Staphylococcus aureus*, and tests were carried out with serum as well as with whole blood, the number of organisms implanted in the former case being half that for the latter.

The results of the test showed that the whole blood was unaffected in bactericidal power by the radiation in all cases; in only one case of the four the unheated serum showed after radiation an increase of bactericidal power, and heating the sera at 55° C. for ½ hour abolished this difference.

As a whole, these results indicate that an increase of bactericidal power of whole blood or serum is detectable only exceptionally 5–15 minutes after the first exposure of children for 4–5 minutes to the mercury vapour lamp.

In the radiation experiments carried out on the bactericidal power of the blood of man and animals by Colebrook, Eidinow and Hill (1924) the time of exposure was 30–120 minutes, and the specimen of blood which showed the greatest increase of bactericidal power was that withdrawn ½ to 3 hours after radiation. In the experiments recorded above the treatment was of short duration, because it was the first exposure and the patients were very young. Blood was withdrawn 5–15 minutes after radiation because it was thought that a weak effect produced by a short exposure might pass off in a shorter time than would be required for a more marked effect to reach its maximum; and only one specimen was taken after radiation in consideration of the age of the patients. For these reasons the significance of this series of results is doubtful, but they are remarkably consistent and are recorded on that account.

Parallel comparative tests of the content of the serum in hæmolytic complement were also carried out, but no change was found after radiation.

### (B) *Rabbits Exposed to the Mercury Vapour Lamp.*

In these experiments two precautions were taken to obviate the influence of other factors on the bactericidal power of the blood: First, the abdomen of the animal was shaved 18–24 hours before exposure, as advised by Colebrook,

Eidinow and Hill (1924), to avoid the possible photo-sensitizing effect of a depilatory substance such as barium sulphide, and to allow the erythema caused by shaving to subside; and second, the animal was allowed access to food before and after radiation in view of the possibility that deprivation of food for some hours might introduce another disturbing factor.

*Experiment.*—Rabbit exposed to mercury vapour lamp for ½ hour at 1 ft. distance.

| | Whole blood (average of 4 tests). | Leucocyte count. |
|---|---|---|
| Immediately before radiation | 18 | 10,100 |
| ½ hour after radiation | 5 | 11,800 |

In this case there was a marked rise in bactericidal power without great variation in the leucocyte count.

*Experiment.*—Another rabbit similarly exposed.

| | Whole blood. 1st test (average of 4 tests). | Whole blood. 2nd test. | Leucocyte count. |
|---|---|---|---|
| Immediately before radiation | 30·5 | 44 | 6,800 |
| „ after „ | 35·5 | ... | 6,200 |
| ¾ hour „ „ | 33 | ... | 6,200 |
| 2½ hours „ „ | 15 | 12 | 7,000 |
| 5 „ „ „ | 33 | ... | 4,500 |
| 29 „ „ „ | 29 | ... | 4,700 |

The bactericidal power of the specimens taken after radiation was very nearly equal to that of the specimen taken before radiation, except the specimen taken after 2½ hours, which was markedly more bactericidal than the others.

*Experiment.*—Another rabbit was exposed for 15 minutes at 20 in. distance. There was a slight decrease in bactericidal power of whole blood at the end of 1 hour and a definite increase after 6½ hours.

*Experiment.*—A rabbit was exposed to the lamp for ½ hour at a distance of 1 ft., and a specimen of defibrinated blood was similarly exposed to radiation under a thin sheet of quartz. In this case *Staphylococcus aureus* and *B. coli* were used in separate tests of the whole blood.

| | Whole blood (*S. aureus*). | Whole blood (*B. coli*). | Leucocyte count. |
|---|---|---|---|
| Immediately before radiation | 39 | 21 | 7,600 |
| ½ hour after exposure of animal to radiation | 20 | 12 | 4,630 |
| After 2½ hours | 28 | 12 | ... |
| 2½ hours after radiation of blood *in vitro* | 30 | 25 | .. |

There was a considerable increase of bactericidal power in the specimens taken after radiation, both for staphylococci and *B. coli*. In the case of the blood exposed *in vitro* there was no definite alteration. This result is in agreement with that of a similar experiment recorded by Colebrook, Eidinow and Hill (1924).

Two rabbits were exposed to ultra-violet radiation for ½ hour at 1 ft. distance. In one case an apparent decrease in bactericidal power of whole blood ½ hour after radiation was followed 2 hours later by restoration to nearly the value immediately before radiation; in the other case there was no significant change in the bactericidal action ¾ hour after exposure.

In two out of these six experiments on rabbits exposed to the mercury-vapour lamp no definite increase in bactericidal power of the blood was demonstrated. In the other four cases a well-marked increase was found at some period or other after radiation. The increase was not maintained in experiments where three or more specimens were tested. The variations which were found in the leucocyte counts offer some evidence that the radiation produced a reaction on the part of the animal organism, but the change in leucocyte count does not always correspond in direction or extent with changes in the bactericidal power of the blood.

(c) *Tests of the Blood of Man and Animals Injected with Various Non-specific Substances.*

The agents used were peptone, bouillon, sodium nucleinate (dissolved in saline and in bouillon) and colloidal manganese. Tests were also made on a person under treatment with sanocrysin. Unless otherwise stated the organism used was *Staphylococcus aureus*, and each figure given is the average of four tests.

For control purposes an animal was injected intravenously with normal saline, and tested in the experiments with other substances.

*Experiment.*—A rabbit was injected intravenously with 1·5 c.c. of sterile physiological saline.

| | Whole blood. | Serum (unheated). |
|---|---|---|
| Immediately before injection . . . | 10 | 14 |
| 3 hours after injection . . . . | 10·5 | 14 |
| 24 „ „ . . . . | 8 | 14 |

No variations of any significance were noted in this experiment.

*Peptone.*

*Experiment.*—A female patient was injected intravenously with 1·2 c.c. of Armour's peptone, with resulting marked protein shock.

| | Whole blood. | Serum. |
|---|---|---|
| 14 hours before injection . . . | 17 | 22 |
| 20 minutes after „ . . . | 17 | 19 |
| 6 hours „ „ . . . | 2 | 20 |

In this case the first specimen was withdrawn the evening before the day of injection.

No change was found 20 minutes after injection compared with the previous evening, but after 6 hours a marked increase was noted.

The serum showed no significant change.

*Experiment.*—A female patient was injected intravenously with 2 c.c. of Armour's peptone. At the end of 14 hours only a very slight and insignificant

variation of bactericial power was observed, and a decrease in leucocyte count from 8000 per c.mm. to 6400.

*Bouillon.*

*Experiment.*—A rabbit was injected intravenously with 1·5 c.c. of nutrient bouillon, as prepared for bacteriological purposes.

| | Blood. | Serum (unheated). |
|---|---|---|
| Immediately before injection . . . | 9 | 52 |
| 3 hours after injection . . . . | 2 | 79 |
| 20 „ „ . . . . . | ... | 63 |

An apparent increase in the bactericidal power of whole blood occurred.

*Sodium Nucleinate 5% in Saline.*

*Experiment.*—A rabbit was injected intravenously with 1·5 c.c. of a 5% sterile solution of sodium nucleinate in saline.

| | Blood. | Serum (unheated). |
|---|---|---|
| Immediately before injection . . . | 25 | 54 |
| 3 hours after injection . . . . | 9 | 55 |
| 20 „ „ . . . . . | 27 | 58 |

An increase of the bactericidal power of the blood 3 hours after injection was followed by a return of the original value 20 hours after injection. No change was found in the case of serum.

*Sodium Nucleinate 5% in Bouillon.*

*Experiment.*—A rabbit was injected intravenously with 1 c.c. of a 5% sterile solution of sodium nucleinate in bouillon.

| | Blood. | Serum (unheated). | Leucocyte count. |
|---|---|---|---|
| Immediately before injection . . | 9 | 25 | 5,200 |
| 3½ hours after injection . . . | 1·5 | 15 | 10,500 |

A marked increase of bactericidal power of the blood and serum is demonstrated, with an increase of leucocytes.

Each of two rabbits was injected intravenously with 1·5 c.c. of the same solution of sodium nucleinate in bouillon. In one a very marked increase of bactericidal power of the blood was noted without alteration as regards the serum, and without great variation in the leucocyte count. In the other a considerable increase of bactericidal power of the blood occurred 3 hours after the injection, followed by a decrease in 20 hours; the serum showed less variation.

*Colloidal Manganese.*

*Experiment.*—A rabbit was injected intravenously with 1 c.c. of a commercial preparation of colloidal manganese.

| | Blood. | Serum (unheated). | Leucocyte count. |
|---|---|---|---|
| Immediately before . . . . | 24 | 25 | 7,500 |
| 3½ hours after . . . . . | 12 | 20 | 7,300 |

A definite increase of bactericidal power was seen in the case of whole blood, while the leucocyte count showed no significant change.

In a further experiment with colloidal manganese no appreciable change occurred.

*Ox Serum.*

*Experiment.*—A rabbit was injected subcutaneously with 20 c.c. of fresh ox serum. The blood was tested with *Staphylococcus aureus* and *B. diphtheriæ*.

| | Blood (*S. aureus*). | Blood (*B. diphtheriæ*). | Leucocyte count. |
|---|---|---|---|
| Immediately before | 30·5 | 22·5 | 15,600 |
| 18 hours after | 4·5 | 13 | 11,500 |

A considerable increase of bactericidal power towards the staphylococcus was found. The increase of bactericidal action to *B. diphtheriæ* was less marked. There was no associated leucocytosis. This result is of interest in view of certain observations made by Mackie (1923), who found that in guinea-pigs experimentally infected with *B. diphtheriæ*, the normal serum of the ox and certain other animals exerted a non-specific protective action when injected subcutaneously at the same time as the inoculation.

*Sanocrysin.*

*Experiment.*—A female patient was injected intravenously with sanocrysin. A mild reaction, clinically satisfactory, was produced.

| | Blood. | | | Serum. | Leucocyte count. |
|---|---|---|---|---|---|
| | 1st test. | 2nd test. | 3rd test. | | |
| Immediately before | 15 | 35·5 | 55 | 47 | 8,000 |
| 4½ hours after | 6·5 | 14·5 | 38·5 | 44 | 11,000 |

There was a definite increase of bactericidal power of the blood, with no significant change in the serum. A moderate leucocytosis occurred.

## DISCUSSION OF THE VARIATIONS NOTED.

It is apparent that the bactericidal power of the blood varies in normal conditions of healthy life apart from any unusual influences. In the case of the human subject it was found that the bactericidal power was usually greater in the afternoon (3–4.30 p.m.) than at 9 a.m. or 1 p.m., just before meals.

In animals, on the whole, more constancy was found throughout the day than in the case of humans, the variations that occurred being mostly of a less degree.

In some cases no significant change in the bactericidal power of whole blood or serum was found after the application of the various influences tested —ultra-violet radiation, injection of peptone, sodium nucleinate, collosol manganese, sanocrysin, etc.; rarely a fall was recorded; when an increase occurred it was not always in the first specimen examined after treatment,

and usually there was restoration to the original value in subsequent specimens, the increase lasting 2 or 3 to 24 hours.

As to the degree of the increase found, in human cases it was not clearly shown to be greater than the degree of increase which occurred as a normal daily variation, but in rabbits the normal variations were generally less than in the human cases, and on the whole more frequent and more marked changes following treatment were observed than in the human cases.

Three of the human cases were especially interesting, because in each a definite non-specific reaction was manifested clinically after the injection of the peptone or sanocrysin.

## EXPERIMENTS ON THE MECHANISM OF THE BACTERICIDAL ACTION OF WHOLE BLOOD AND THE FACTORS CONCERNED IN ITS VARIATION.

It may be supposed that the total bactericidal action of whole blood depends on the summation of different properties, and that variations may be the result of alterations in any one or more of these. Thus, the phagocytic action of the leucocytes along with the normal non-specific opsonin probably plays an essential part in the process, while in the case of certain organisms the normal bactericidal principle of the serum by itself may be partly responsible for the effect. Further, any variation in the leucocyte content of the blood and quantitative changes in the opsonic and bactericidal principles of the serum would bring about marked changes in the total bactericidal action of the blood. Even if the effect is mainly due to the phagocytosis, this property is dependent on the serum opsonins.

With a view to throwing some light on the factors concerned in the process the following experiments were carried out:

*Experiment.*—A specimen of blood was taken from a rabbit and divided after defibrination into two equal parts. The serum and corpuscles of each moiety were separated by centrifuging, and the serum of one moiety was heated for ½ hour at 55° C., while the serum of the other moiety was kept at cool room temperature for the same time. The sera were then added to the respective cells again and the reconstituted blood specimens were tested.

| | Staphylococcus (average of 4 tests). | *B. coli* (average of 4 tests). |
|---|---|---|
| Cells plus heated serum . . . . | 9 | 63 |
| „ unheated serum . . . . | 1 | 16 |

*Experiment.*—Two exactly similar experiments performed with other specimens.

| | Staphylo-coccus. | Staphylo-coccus. |
|---|---|---|
| Cells plus heated serum . . . . | 37 | 66 |
| „ unheated serum . . . . | 21 | 41 |

The results of these experiments clearly show that the bactericidal effect of whole blood is dependent on thermolabile constituents of the serum, such as the normal thermolabile opsonin or the bactericidal complement.

*Experiment.*—A specimen of human blood was withdrawn and various

changes in the proportion and condition of the serum were made; blood specimens were reconstituted as in the previous experiments after the corpuscles had been washed with sterile physiological saline. The numbers of colonies developing in the reconstituted bloods were compared with the number in the original blood and in various media.

*Staphylococcus aureus* was the organism implanted.

| | Colony count (average of 4 tests). | Leucocyte count. |
|---|---|---|
| 1. Whole blood unchanged | 14 | 4,800 |
| 2. Reconstituted blood. Cells plus serum, unheated | 27 | ... |
| 3. Reconstituted blood. Cells plus serum, heated | 91 | ... |
| 4. Reconstituted blood. Cells plus ½ original proportion of unheated serum plus equal volume of saline | 22 | ... |
| 5. Cells plus volume of saline equal to serum | 60 | ... |
| 6. *Reconstituted blood. Cells with larger proportion of leucocytes than normal plus unheated serum in normal proportion to corpuscles | 12 | 5,700 |
| 7. Serum, unheated | 122 | ... |
| 8. Serum, heated | 126 | ... |

* To prepare this specimen corpuscles and serum were separated by centrifuging, and a certain amount of the upper layers of the corpuscles, including more whites than the lower layers, was added to serum in the same proportion as in the original blood.

The unmanipulated specimen of blood showed greater bactericidal power than the specimen reconstituted with corpuscles and unheated serum in the original proportions, so that the latter must be the basis for comparison of the other reconstituted specimens. Unheated serum alone had apparently little bactericidal power since the colony count corresponded closely to that in heated serum. As in previous experiments, the bactericidal power of the whole-blood specimen containing heated serum was considerably reduced as compared with that containing fresh serum.

It might be concluded from these findings that the bactericidal action of blood is due to the combined action of leucocytes and a thermolabile constituent of the serum. Replacement of half of the unheated serum by saline made no significant difference to the bactericidal power of the blood, suggesting that the amount of the thermolabile element present originally in the serum of this specimen was in excess. When the proportion of leucocytes in the corpuscles was artificially increased (by centrifuging them and pipetting off the upper layers) and serum was added to the corpuscles in the same proportion as normal, there was a marked increase in the bactericidal power of the product—which illustrates the influence of the leucocyte content of the blood and the degree of its bactericidal properties.

## DISCUSSION OF INFORMATION OBTAINED AS TO THE FACTORS CONCERNED IN THE BACTERICIDAL ACTION OF BLOOD.

The great advantage of the slide-cell method is that corpuscles and sera are tested together in their normal proportions and as little disturbed by manipulations as possible. It is not surprising to find that serum alone is much less bactericidal than whole blood, and that the factor of phagocytic elimination of organisms plays an essential part in the process. This is reflected in the practice adopted of using for serum, implantations half as large as for whole blood.

It was found fairly constantly that implantation of *Staphylococcus aureus* in the unheated serum of normal persons and animals yielded practically the same colony count as in serum heated at 55° C. for ½ hour. If it is assumed that heated serum *per se* possesses no bactericidal action owing to the inactivation of complement, then fresh normal serum is also devoid of any direct bactericidal action for this organism. This deduction is in line with the belief commonly held in regard to normal human serum that it has no bactericidal power against the staphylococcus. Thus bactericidal complement in this case would play little part in the normal process. When comparison was made between specimens of unheated serum obtained from a person or animal before and after various forms of treatment, there were marked differences in some cases. An apparent acquisition of bactericidal power after treatment was found in three experiments. It was noted that alterations in the bactericidal power of whole blood and serum were usually, but not always, parallel. Colebrook, Eidinow and Hill (1924) stated that, generally speaking, if the whole blood in the slide-cells showed alteration, a similar result was also observed in the serum, but the latter was manifested a little later, *e.g.* blood might show a maximal effect after 1 hour and serum not until 2 hours. My results do not permit me to state such a definite time relationship between the activation of the blood and serum.

By means of experiments with reconstituted bloods several observations were made. It was found that separation of corpuscles and serum and re-mixing them caused some loss of bactericidal power. When a specimen of blood was divided into two equal parts, and the corpuscles and serum separated, the moiety reconstituted with fresh serum was markedly more bactericidal than the one with heated serum, showing that the heating of the serum had destroyed some property (probably that of the labile opsonin) contributing to the elimination of the implanted bacteria, though not capable of functioning independently. It may be noted that in a few cases in which the hæmolytic complement content of unequally bactericidal bloods was tested, there was no difference in the minimum hæmolytic dose.

In regard to leucocytosis no constant relationship can be detected from the results between increase or decrease of bactericidal power and the rise and fall of the leucocyte count of the peripheral blood. In certain experiments in which there was an increase in the bactericidal power of the blood there was a coincident rise in the leucocyte count, but in some cases an increase in the bactericidal power was accompanied by no change or a decrease in the leucocyte count; in other cases the leucocytes were increased and the bactericidal power remained unaltered. It may be noted that the blood for

leucocyte counts was always withdrawn within 3–5 minutes before or after the blood for the bactericidal tests. The reference already made to Shaw's work (1927) on the diurnal leucocyte tides is repeated here particularly in regard to the alterations found in leucocyte counts of untreated persons and animals, and also to suggest a possible analogy between the leucocyte tides and the diurnal changes of the bactericidal power of the blood.

It will be seen that the variations in the bactericidal action of the whole blood cannot be fully correlated with changes in the leucocyte content or the bactericidal effect of the serum *per se*, and the processes involved are probably of more complex nature than can be explained in terms of phagocytosis, opsonins and bactericidal complement. No work was done in regard to the ferments of the blood but the possibility of their participation in producing changes in the bactericidal power of the blood must be kept in mind in view of the work on the blood ferments by Jobling, Petersen (1922), and others.

## CONCLUSIONS AND SUMMARY.

1. The slide-cell method of testing the bactericidal power of whole blood is a reliable experimental method, and the results obtained are consistent within fairly appreciable limits.

2. The bactericidal power of the blood of healthy persons and animals exhibits diurnal variations.

3. The apparent changes produced in the bactericidal power of the blood of man and animals by certain agents are transient. In man such changes are not of much greater extent than the normal diurnal changes. In animals more marked alterations, often greater than the usual diurnal variations, followed exposure to ultra-violet radiation and the intravenous injection of bacteriological bouillon, sodium nucleinate, collosal manganese and normal ox-serum.

4. Some observations have been made on the factors concerned in the bactericidal process of whole blood and its variations.

---

It gives me great pleasure to acknowledge my indebtedness to certain clinicians for their advice and assistance in obtaining specimens, particularly to Prof. D. Murray Lyon and Dr. T. Y. Finlay. My thanks are also due to Prof. T. J. Mackie for the interest he has taken in the work and the advice given at every stage.

## REFERENCES.

COLEBROOK, L., EIDINOW, A., AND HILL, L.—(1924) *Brit. J. Exp. Path.*, **5**, 54.
COLEBROOK, L., AND STORER, E. J.—(1924) *Ibid.*, **5**, 47.
FLEMING, A.—(1926) *Ibid.*, **7**, 274.
MACKIE, T. J.—(1923) *J. Immunol.*, **8**, 35.
MAITLAND, H. B.—(1926) *Brit. J. Exp. Path.*, **7**, 161.
PETERSEN, W. F.—(1922) 'Protein Therapy and Non-Specific Resistance.' The Macmillan Co., New York. Chap. 3, pp. 64–69; Chap. 6, pp. 102–127, etc.
SHAW, A. F. B.—(1927) *J. Path. Bact.*, **30**, 1.
WALSH, V. G., AND HARMSWORTH, D.—(1926) *Brit. J. Exp. Path.*, **7**, 129.
WRIGHT, A. E., COLEBROOK, L., AND STORER, E. J.—(1923) *Lancet*, **1**, 365, 417, 473.

# THE EXPERIMENTAL TRANSFORMATION OF THE INTESTINAL FLORA.

ROBERT CRUICKSHANK, M.D.Aberd., D.P.H.

*From the Bacteriology Department, University of Aberdeen.*

Received for publication August 24th, 1928.

*Definition of terms.*—The majority of organisms whose natural habitat is the intestine are *saccharolytic* (carbohydrate-splitting) or *proteolytic* (protein-splitting), or both; and bacteria possessing both saccharolytic and proteolytic powers, for example, the coliform group and certain anaerobes, utilize any available carbohydrate before attacking the protein moiety. *Saccharolytic* intestinal organisms are, according to Kendall and Haner (1924), *B. acidophilus* and *B. bifidus*, *B. welchii* and the enterococcus: consequently a saccharolytic or alternatively a *fermentative* flora should be representative of all these organisms. However, the conditions which favour the production of a fermentative flora apparently afford the optimum environment for the growth of the acid-tolerant—or aciduric—group of organisms (type, *B. acidophilus*) so that these tend to dominate the picture and a fermentative becomes an *aciduric* flora. *B. welchii* is rarely recovered from such a flora and the enterococcus is but sparsely represented: *B. coli* can usually be isolated. It is more difficult to define a proteolytic or *putrefactive* flora. Among the proteolytic organisms which may be present in the intestine are *B. sporogenes*, *B. putrificus*, *B. mesentericus*, *B. histolyticus*, *B. proteus* and *B. pyocyaneus*: in addition there are the bacteria playing a dual *rôle*, and it is not unlikely that the coliform group, forming as it does a major proportion of the "normal" flora, contributes to intestinal proteolysis. If a flora is proteolytic which yields all or most of these organisms, probably 90 per cent. of the adult population would be included in the category of those harbouring a putrefactive, and professedly harmful, flora. The term "*facultative*" flora has therefore been suggested for the "normal" adult flora and a putrefactive flora defined by the marked proteolysis produced by its constituent organisms on certain media (Morris, Porter and Meyer, 1919). These authors admit that the difference between a facultative and a putrefactive flora is only one of degree. It is an interesting phenomenon that the spores of *B. welchii* are present in greater abundance in a proteolytic than in a fermentative, or even a normal, flora (Kahn, 1924; Kendall, Day and Walker, 1926).

*Historical.*—The use of diets rich in carbohydrate to control putrefactive activity in the gut is not a recent development or discovery. In 1886, Hirschler fed dogs with cane sugar, potatoes, glycerol and dextrin, and estimated that as a result the fæces contained indol and phenol in less amounts than when meat alone was given. Winternitz (1892) concluded from animal experiments that feeding with milk lessens putrefaction, causing a decrease in the production of ethereal sulphates. He regarded the beneficial results as being due to the lactose in the milk independent of the lactic acid produced. Herter and

Kendall in 1908 were probably the first workers to examine the relationship of the chemical nature of the food to the intestinal flora. They tested the effect of various diets on the intestinal flora of cats and monkeys, and concluded that a high carbohydrate diet following a diet of meat and eggs tends to eliminate the putrefactive bacteria and to substitute instead a fermentative flora. Jungano (1909) found that rats fed on a meat diet developed a markedly putrefactive flora with predominance of *B. coli*. In 1914 Distaso and Schiller fed white rats with lactose and dextrin, added to an ordinary diet of bread and meat, and reported a rapid transformation of the intestinal flora to the aciduric type. They attributed the change to the slow absorption of these carbohydrates (lactose and dextrin), so that they reached the lower intestine where they afforded a suitable pabulum for the growth of the fermentative bacteria. About the same time, and independently, Hull and Rettger tested the effect of various carbohydrates (lactose, maltose, sucrose, dextrose, lævulose, galactose and dextrin) on the intestinal flora of white rats, and found that lactose alone was capable of producing a change from a proteolytic to a fermentative flora. Further experiments by the same authors (1917) confirmed their earlier findings, and, in addition, milk and mixed grains caused an increase of the aciduric organisms. Torrey (1919) using dogs, because their intestinal flora more closely resembled that of man, conducted a large series of feeding experiments not only with the special sugars, but also with various dietaries rich in carbohydrate, protein and fats respectively. He showed that the two carbohydrates lactose and dextrin, when added to a diet of meat and rice (taken as the normal diet), caused such a marked development of aciduric bacteria that these organisms dominated the intestinal flora and effected an almost complete suppression of proteolytic organisms including *B. coli*. This purely fermentative flora continued so long as the special sugar was fed. Glucose, saccharose and maltose caused an increase in the numbers of streptococci but failed to produce any transformation to an aciduric flora. Starchy foods, particularly potatoes, white bread and beans tended towards a simplification of the flora. Of the proteins, animal proteins (flesh and fish) produced a markedly proteolytic flora, milk casein was much less active, while vegetable proteins offered no encouragement to the development of a putrefactive flora. Fat as an element of diet had no effect on the intestinal flora. In 1921, Rettger and Cheplin published a treatise on the transformation of the intestinal flora of white rats and man with special reference to the implantation of *B. acidophilus* by the administration of the viable organism, a procedure first suggested by Rotch and Kendall (1911). As regards the various sugars, they confirmed the findings of previous workers. In addition they showed that emulsions in saline of *B. acidophilus*, or "acidophilus-milk," changed the intestinal flora of rats and of man to the aciduric type, while a combination of lactose or dextrin with an emulsion of *B. acidophilus* was equally efficacious. *B. bulgaricus* even when given in large doses failed to produce any change in the flora, and was unable under any circumstances to establish itself in the alimetary canal of albino rats. These results have been generally confirmed by other workers. Sissoon (1917), however, stated that a milk diet containing 10 to 15 per cent. of lactose did not produce an intestinal flora differing essentially from that following a diet of pasteurized milk. Cannon (1921) found that in experimental animals

vegetable proteins not only reduced the relative proportion of proteolytic types (aerobic and anaerobic), but also encouraged the growth of organisms which did not form gas.

## THE TRANSFORMATION OF THE INTESTINAL FLORA OF RATS.

It was thought desirable to carry out a series of feeding experiments on rats with the following aims in view:

1. To substantiate the findings of Rettger and Cheplin, and others, on the relationship existing between the chemical nature of the dietary and the intestinal flora: in particular, to study the effect of certain carbohydrates in changing the flora to the fermentative or aciduric type.

2. To find out what substance or combination of substances produces most quickly and most completely an aciduric flora in the intestine.

3. To attempt the implantation of *B. acidophilus* in the intestine by the use of emulsions of the organism or by milk fermented with *B. acidophilus* (so-called "acidophilus-milk"): to determine how and why acidophilus-milk effects a transformation of the flora to the aciduric type. The action may be a physical one due to the mere bulk of milk. It may be chemical due to the lactic acid or other products of fermentation of the milk. Or it may be wholly biological, requiring only the presence of viable *B. acidophilus*. Kopeloff and Beerman (1924), by experiment on the human individual, concluded that the viable organism is the essential factor. The rat was used in the present series of experiments as a more suitable test-subject: it is more easily controlled and the intestinal flora readily responds to changes in the dietary.

### *Method.*

Four rats were used for each series of experiments. They were kept on a basic diet of bread 10 gm., meat 3 gm. and oats for a week previous to the commencement of each feeding experiment during which time their fæces was examined at two-day intervals to determine the normal flora. Each substance being tested was added to the basic diet during a period of fourteen or more days and the fæces again examined every three days to find out what change, if any, the substance had produced on the intestinal flora. The fæces was received into a sterile test-tube by holding the rat up by the tail, 0·2 gm. (not weighed) was emulsified in 2 c.c. of sterile saline and films stained by Gram's method. A differential count of the Gram-negative and Gram-positive organisms was done and a rough estimate of the relative numbers of *B. acidophilus* and Gram-positive cocci made. Deep tubes of liquefied glucose-agar, casein-agar or liver-glucose-agar (Torrey) were inoculated with a small loopful of the fæcal emulsion, poured in Petri capsules and incubated aerobically at 37° C. for 48 hours. The colonies of *B. acidophilus* are quite distinctive in any of these media* and the proportion of these to other types of colony controls the estimate of the percentage of *B. acidophilus* made from the differential count on the direct smear. Tubes of acid broth were also inoculated heavily with the fæcal emulsion and acidophilus bacilli isolated by the method of Cruickshank and Berry (1924). Where *B. acidophilus* was being fed to the rats, either in

* *B. acidophilus* can be cultured more easily from the animal-intestine than from the fæces of man and grows readily by direct sowing in solid media.

emulsion or in milk, agglutination tests were carried out with the strain of *B. acidophilus* isolated from the fæces against an antiserum prepared to the strain which was used in the preparation of the milk or the emulsions. Almost without exception the organism recovered from the fæces was agglutinated in those experiments in which viable *B. acidophilus* were used in the attempt to transform the flora to the aciduric type, but not otherwise. It suggests that the particular strain of *B. acidophilus* being used had become "implanted" in the intestine to the exclusion of other strains which are normally present.

The effect of a high carbohydrate and a high protein diet on the intestinal flora was first determined and the following substances were then used in the experimental attempts to produce an aciduric flora:

(*a*) *Carbohydrates:* Dextrin, lactose, and the monosaccharides glucose and galactose to which lactose is split, saccharose, maltose and lævulose.

(*b*) Emulsions of *B. acidophilus*, alone or in combination with lactose or dextrin.

(*c*) Acidophilus-milk*; and the following modifications: (1) The same bulk of unfermented milk; (2) the curd of acidophilus milk separated from the whey by filtration and containing the viable organisms; (3) the whey of acidophilus milk; (4) milk treated with lactic acid or with hydrochloric acid to produce the same degree of acidity as is present in acidophilus-milk. (The acidity was estimated by titration with N/1 NaOH.) (5) Acidophilus-milk heated at 100° C. for 10 minutes. In addition the combined effect of acidophilus milk and lactose was tested.

### *Results.*

These are perhaps best represented by graphs showing the percentage of *B. acidophilus* present in the fæces during the period of the experiment (Charts 1–5). Only those experiments which resulted in a change to or towards an aciduric flora need be shown: for the rest, it is sufficient to state that of the carbohydrates maltose, glucose, galactose and lævulose produced no change in the flora. Lactose was more effective than dextrin perhaps because the animals seemed to take it more readily. Saccharose produced some simplification of the flora. Emulsions of *B. acidophilus* failed to effect any change but the amount used was considerably less than that with which Rettger and Cheplin were able to induce an aciduric flora. The addition to the bacterial emulsion of 1 gm. of lactose—itself insufficient to cause any change—resulted in an appreciable increase of *B. acidophilus* in the flora. Acidophilus-milk was quickly effective in changing the flora to the aciduric type and in combination with lactose even more so. An equal bulk of unfermented milk, milk acidified with lactic acid or with hydrochloric acid, acidophilus-milk heated at 100° C. for 10 minutes to kill the organisms, all failed to produce any change in the flora. The curd of acidophilus-milk, on the other hand, containing viable organisms and some unfermented sugar, changed the flora to a fermentative type, whereas the whey alone had no effect.

* The method used in the preparation of acidophilus-milk was that described by Rettger and Cheplin.

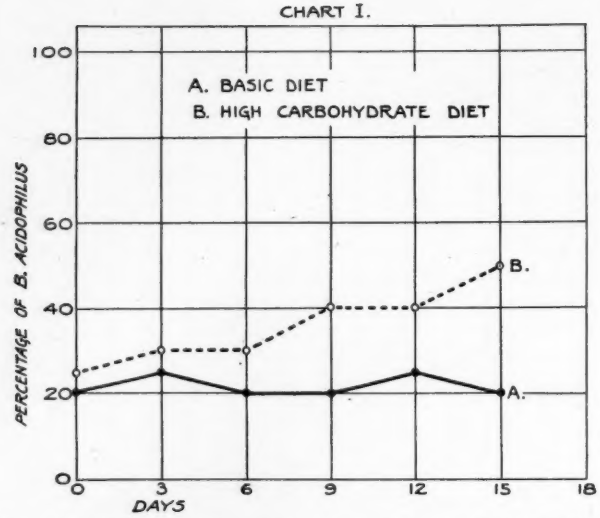



CHART I: A. *Basic diet* of bread, 10 gm., meat 3 gm. and oats. *Fæces* hard and slightly constipated. *Flora:* heterogeneous: *B. coli* abundant, spore-bearers present, *B. acidophilus* scanty.

B. *High carbohydrate diet:* bread 10 gm., milk 10 c.c. *Fæces* well-formed: not constipated. *Flora:* simplified: increase of *B. acidophilus*.

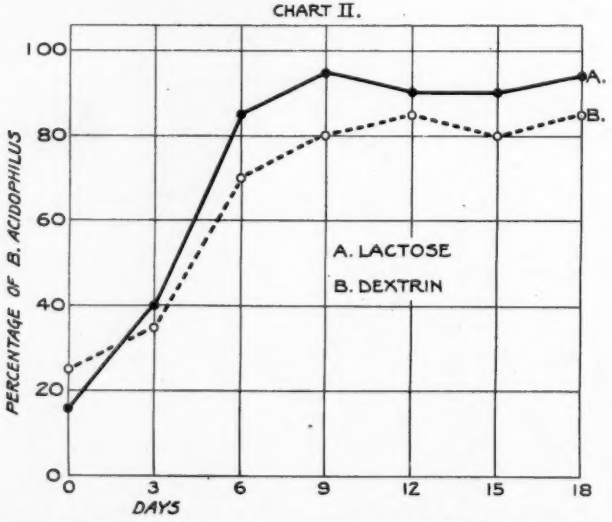



CHART II: A. Basic diet plus *lactose* 4 gm. *Fæces* soft and light brown in colour: slight diarrhœa in one rat. *Flora:* quickly transformed to aciduric type. *B. coli* scanty: spore-bearers present.

B. Basic diet plus *dextrin* 4 gm. *Fæces* soft: not constipated. *Flora:* gradually changed to "fermentative" type. Dextrin is not very palatable to man and the rats did not take it well.

CHART III.

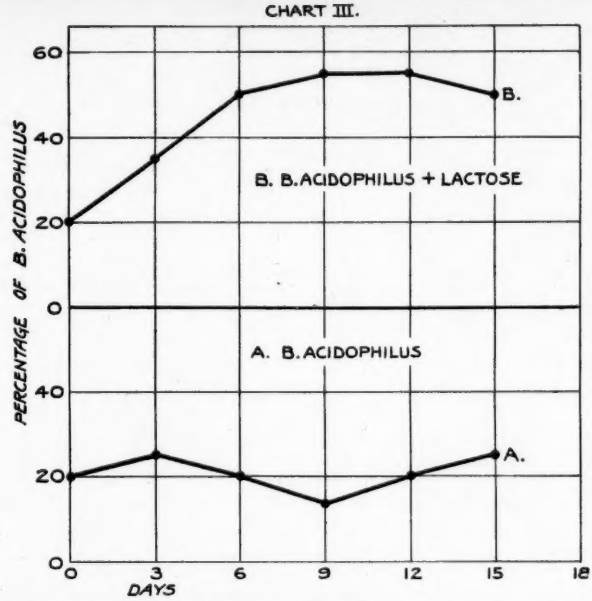



CHART III: A. Basic diet plus emulsion of *B. acidophilus* (prepared from three 48-hour cultures on agar slopes). *Flora:* no appreciable change.

B. Basic diet *B. acidophilus* plus lactose 1 gm. *Flora:* less "mixed": definite increase of *B. acidophilus*. One gram of lactose added to the basic diet failed to influence the flora. In both experiments, *B. acidophilus* recovered from fæces was agglutinated by antiserum to strain being administered.

CHART IV.

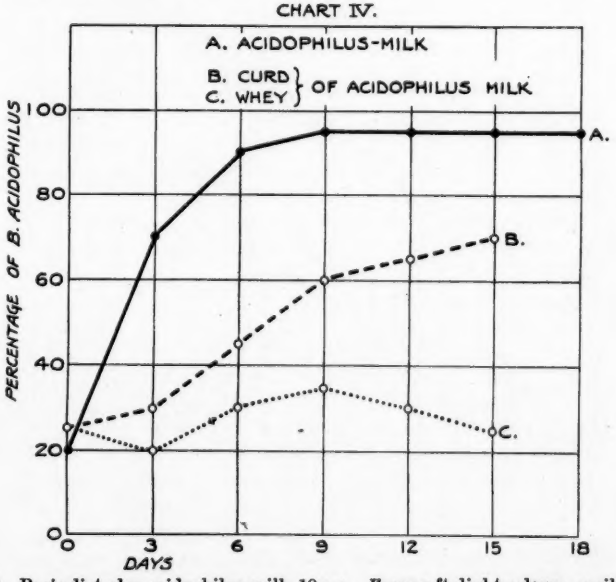



CHART IV: A. Basic diet plus acidophilus-milk, 10 c.c. *Fæces* soft, light colour: easily passed. *Flora:* rapidly changed to aciduric type. Many colonies of *B. acidophilus* isolated from fæces were tested and agglutinated by antiserum to strain used in preparation of the milk.

B. Basic diet plus 10 c.c. of curd* containing living *B. acidophilus*. *Flora:* changed to fermentative type.

C. Basic diet plus 10 c.c. of whey from acidophilus-milk. *Flora:* no change.

* There was a marked reduction of Fehling's solution by a watery extract of the curd.

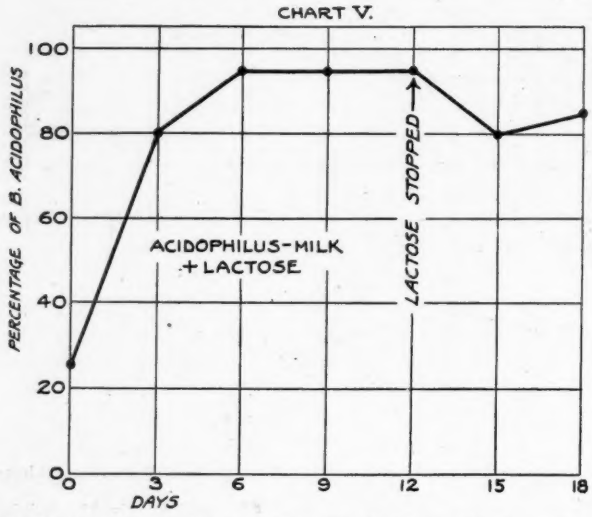



CHART V: Basic diet plus 10 c.c. of acidophilus-milk plus 2 gm. of lactose. *Fæces* soft: no diarrhœa. *Flora:* rapidly became aciduric. *B. coli* scanty: spore-bearers not recovered.

## DISCUSSION.

Lactose, a disaccharide and dextrin, a polysaccharide are the only two carbohydrates capable of effecting a complete transformation of the intestinal flora to an aciduric type. Rettger and Cheplin have shown that in rats fed with lactose or dextrin reducing bodies may be demonstrated in the cæcum and colon as well as in the fæces, and they deduced that these two carbohydrates are absorbed relatively slowly from the intestinal tract. The cæcum and colon are the great breeding grounds of the intestinal bacteria and this unusual supply of carbohydrate is probably attacked alike by acidophilus, coliform and other saccharolytic bacteria with the production of much acid. As the concentration of acid increases—and Cannon and McNease (1923) have shown that the hydrogen-ion concentration of the contents of the cæcum in rats fed on lactose is 4·4 compared with a pH of 6·2 in the controls—the environment becomes unfavourable for all organisms save the acid-tolerant group with the consequent appearance of an aciduric flora. This explanation is in agreement with *in vitro* experiments which show that *B. coli* fails to grow in a medium whose pH is more acid than 5·0 and does not survive if grown in association with *B. acidophilus* or *B. bifidus* in a carbohydrate-containing medium (Cruickshank, 1925): proteolysis by spore-bearing anaerobes is also inhibited at pH 5·6 (Torrey and Kahn, 1923). Moreover, the suckling infant on a diet rich in lactose has an entirely aciduric flora and the fæces passed quickly along the gut is distinctly acid: for example, the stools of twenty breast-fed infants gave an average pH of 5·2.

Rettger and Cheplin believe that the aciduric flora is established by the mechanical crowding out of other organisms by the proliferation of *B. acidophilus* on a medium particularly favourable to its growth. They were led to this conclusion mainly because there was no increased acidity of the fæces even when the flora was wholly aciduric. But as Robinson (1922) has pointed out this is due to the change from acid reaction towards neutrality as the contents pass along the large gut. In diarrhœal conditions the stools are more acid.

The feeding experiments with acidophilus milk and emulsions of *B. acidophilus* lead one to conclude that the important factor is neither a physical one due to the *bulk* of milk nor chemical due to the *acidity* of the milk but that both the viable organism and available carbohydrate are necessary to effect a radical transformation to an aciduric flora. Emulsions of the organism alone have been stated to produce the change (Rettger and Cheplin, Gompertz and Vorhaus, 1923), but it is difficult to understand how *B. acidophilus* can become "implanted" and maintain itself as the predominant organism in the intestine without the presence in the cæcum and colon of utilizable carbohydrate such as lactose and dextrin can supply. The quick return of the flora to normal as soon as the administration of lactose is discontinued bears out this contention. In an experiment on oneself, large quantities of acidophilus-milk (one litre a day, freshly prepared) in combination with lactose (10 oz. divided into two doses) were required to produce a simplification of the flora, by no means wholly aciduric. Either substance by itself, in the doses mentioned, failed to effect any marked change in the flora. The conclusion derived from animal experiment that acidophilus-milk plus lactose effects the transformation of the normal to an aciduric flora most quickly and completely is thus borne out.

## SUMMARY.

1. A diet with a high carbohydrate content produced a simplified flora in the intestines of rats but failed to effect a radical transformation to an aciduric flora.

2. Lactose and dextrin when added to a diet of bread, meat and oats quickly changed the intestinal flora to aciduric type. Saccharose had a much less marked effect: maltose, glucose, galactose and lævulose produced no change.

3. Acidophilus-milk caused a transformation from normal to an aciduric flora in a comparatively short time. In combination with lactose the change was even more rapid and complete. The effectiveness of acidophilus-milk is due not to its bulk or its acidity but to the combination of the living organism with the unfermented lactose in the milk.

4. Under the foregoing conditions, *B. acidophilus* becomes established as the predominant organism in the intestine probably because of its acid-resistant property. Lactose and dextrin are slowly absorbed from the intestine and the attack on available carbohydrate by the intestinal bacteria in the lower bowel produces a degree of acidity which is inimical to all save the acid-tolerant group.

REFERENCES.

CANNON, P. R.—(1921) *J. Infect. Dis.*, **29**, 369.
CANNON, P. R., AND MCNEASE, B. W.—(1923) *Ibid.*, **32**, 175.
CRUICKSHANK, J., AND BERRY, D. W.—(1924) *Brit. Med. J.*, ii, 944.
CRUICKSHANK, R.—(1925) *J. Hyg.*, **24**, 241.
DISTASO, A., AND SCHILLER, J.—(1914) *C.R. Soc. Biol.*, Paris, **73**, 362.
DONALDSON, A. N.—(1922) *J. Amer. Med. Ass.*, **78**, 884.
GOMPERTZ, L. M., AND VORHAUS, M. G.—(1923) *Ibid.*, **80**, 90.
HERTER, C. A., AND KENDALL, A. I.—(1908) *J. Biol. Chem.*, **5**, 293.—(1909) *Ibid.*, **7**, 203.
HIRSCHLER, A.—(1886) *Z. physiol. Chem.*, **10**, 306.
HULL, T. G., AND RETTGER, L. F.—(1914) *Zbl. Bakt.*, I, Orig., **75**, 219.—(1917) *J. Bact.*, **2**, 47.
JUNGANO, N.—(1909) *C.R. Soc. Biol.*, Paris, **66**, 112.
KAHN, M. C.—(1914) *J. Infect. Dis.*, **35**, 423.
KENDALL, A. I., AND HANER, R. C.—(1924) *Ibid.*, **35**, 67, 77, 89.
KENDALL, A. I., DAY, A. A., AND WALKER, A. W.—(1926) *Ibid.*, **38**, 217.
KOPELOFF, N.—(1923) *J. Amer. Med. Ass.*, **80**, 602.
KOPELOFF, N., AND BEERMAN, P.—(1924) *Arch. Intern. Med.*, **33**, 55.
MORRIS, G. B., PORTER, R. L., AND MEYER, K. F.—(1919) *J. Infect. Dis.*, **25**, 349.
RETTGER, L. F., AND CHEPLIN, H. A.—(1921) 'The Transformation of the Intestinal Flora,' Newhaven and London.
ROBINSON, C. S.—(1922) *J. Biol. Chem.*, **52**, 445.
ROTCH, T. M., AND KENDALL, A. I.—(1911) *Amer. J. Dis. Child.*, **2**, 30.
SISSON, W. R.—(1917) *Ibid.*, **13**, 117.
TORREY, J. C.—(1919) *J. Med. Res.*, **39**, 415.
TORREY, J. C., AND KAHN, M. C.—(1923) *J. Infect. Dis.*, **33**, 482.
WINTERNITZ, H.—(1892) *Z. physiol. Chem.*, **16**, 460.

---

# EFFECT OF ULTRA-VIOLET LIGHT ON THE VIABILITY OF THE VIRUS OF FOOT AND MOUTH DISEASE.

I. A. GALLOWAY, B.Sc., M.R.C.V.S.,

AND

A. EIDINOW, M.B., B.S.LOND.

*From the National Institute for Medical Research, London.*

Received for publication September 6th, 1928.

THE bactericidal action of the sun's rays has long been recognized, and its destructive action on the virus of foot and mouth disease has already been recorded by Bedson, Maitland and Burbury (1927). They found that samples of dried virus (known to be still infective) when exposed to direct sunlight (noon in August) for 1 hour were rendered avirulent; even winter light transmitted through glass greatly shortened the period of survival of the virus.

The spectroscopic examination of direct sunlight and scattered light from the sky has shown that infra-red luminous, long and short ultra-violet rays are emitted. The quality and quantity varies to some extent with the atmospheric conditions. With artificial sources of light a more constant type of radiation can be obtained and in this way experimental research can be carefully controlled. The variable conditions under which many experiments have been carried out make it difficult to estimate the value of the results obtained. The suspending fluid or the culture medium, the depth of the layer of fluid irradiated, and the temperature are all important factors.

Many workers have investigated the effects of radiations of various wave-lengths upon micro-organisms in general, and the action of ultra-violet light on viruses belonging to the filter-passing group has also been studied.

Finsen and Dreyer (1903) were the first to show the viricidal effect of light. Their experiments were carried out with a Finsen arc-lamp on vaccinia lymph. Rous (1913) stated that ultra-violet light rapidly killed the cells of his transplantable sarcoma of the fowl, but did not notably injure the ætiological agent associated therewith. Since then, however, the susceptibility of the Rous "agent" to ultra-violet rays has been definitely shown, short exposures completely destroying the infectivity of tumour-filtrates.

Baker and Peacock (1926) have shown that the dose of irradiation necessary to cause complete inactivation of Rous sarcoma-filtrates is considerably in excess of that required to sterilize dilute bacillary emulsions, and this has been confirmed by Illingworth and Alexander (1927).

Early in 1926 Douglas and Eidinow* confirmed Dreyer's observation that ultra-violet light was lethal to the virus of vaccinia. Emulsions of virulent brain were rendered non-infective after 5 minutes exposure to radiations of wave-lengths 5720–2300 Å.U.

McKinley, Fischer and Holden (1926) exposed a strain of bacteriophage and the virus of herpes to ultra-violet radiations, as emitted by an Alpine sun lamp of 4·5 ampères, at a distance of one foot for 40 minutes, in quartz flasks. They found that such ultra-violet light was sufficient to attenuate or destroy both the bacteriophage and the virus of herpes.

Experiments made by Takaya (1926) indicated that both "street virus" and the fixed virus of rabies were weakened by exposure to ultra-violet rays, and that when the rays were strengthened, the virus was killed. Fixed virus showed a stronger resistance to the rays than street virus.

Nicolau and Galloway (1927) found that centrifugalized brain-emulsions of the virus of enzootic encephalomyelitis (Borna disease) were rendered avirulent after 5 minutes exposure in quartz flasks to ultra-violet rays emitted by a mercury vapour lamp, wave-lengths 5720–2300 Å.U. being transmitted.

Since we commenced our experiments, Olitsky and Gates (1927) have published their results as to the effect of ultra-violet light on the viability of the virus of vesicular stomatitis. They used aspirated vesicular contents diluted 1/10 in buffered broth at pH 7·4 as virus. The surface of thin layers of 2% agar buffered at pH 7·4 was washed with this virus. After exposure to irradiation the virus agar was cut within the limits of the area of light

* Personal communication.

penetration, ground in a mortar, suspended in buffered phosphate solution and inoculated intradermally into the metatarsal pads of guinea-pigs.

In our experiments with the virus of foot and mouth disease, our technique was as follows: Two mercury vapour lamps (K.B.B.* type, 2·5 ampères, 210 volts, direct current) were employed, eight inches distant. The flasks containing 5 c.c. of fresh virulent guinea-pig lymph, filtered or unfiltered, and diluted 1/10 in "buffered" saline, pH 7·6 were slowly rotated (being attached to a wheel driven by an electric motor) during the exposure, so that a fresh thin film of fluid was constantly exposed to the lamp. The flasks, dipped periodically into cold water in a trough to prevent overheating during the experiment. The virus used titrated to between 1/500,000 and 1/1,000,000.

In the first series of experiments quartz flasks, which transmit 5720–2300 Å.U. were used, and the time of exposure was for 5, 15 and 30 minutes.

When the lymph was irradiated after filtration through a Mandler filter, the virus was completely destroyed by 5 minutes' exposure in three separate experiments. To ensure absolute sterilization of unfiltered fluid, 30 minutes' exposures were necessary as in one of three experiments, virus could still be demonstrated after 15 minutes' exposure, although in the other two tests it was not detected in samples irradiated for 15 minutes only. This is probably due to absorption of some of the radiations by small suspended particles in the unfiltered fluid. A similar protective action occurred when the virus was suspended in serum during exposure to the ultra-violet rays.† A mixture of equal parts of rabbit serum and filtered virus (diluted 1/10 in phosphate saline, pH 7·6, was irradiated under similar conditions as those obtaining in the previous experiments. The results showed that the virus was protected by the serum when exposure was for 15 minutes only, while after 30 minutes, the virus was destroyed.

In the second series of experiments, the lymph was exposed, filtered or unfiltered, in quartz flasks to the radiations of a mercury-vapour lamp, but filtered through a screen of "vita-glass" (transmits radiations of wave-lengths 5720–2800 Å.U.). The virus was fresh vesicle-lymph diluted 1/10 in phosphate saline, pH 7·6, as in the previous experiments, and exposure to the rays was for 15 minutes, 30 minutes, and 1 hour. Three experiments were carried out under similar conditions, and the results in each case were the same; the virus, filtered or unfiltered, was inactivated after 30 minutes, but not after 15 minutes' exposure to those rays emitted by the mercury arc capable of passing through a screen of vita-glass.

In the third series of experiments, flasks of ordinary glass were used as receptacles for the virus during exposure to the radiations from the mercury-vapour lamps (radiations of wave-lengths 5700–3300 Å.U.).

The virulent lymph, diluted 1/10 as above, was irradiated both in the filtered and the unfiltered state. At intervals after 5, 15 and 30 minutes' exposure samples were removed from the flasks and tested for the viability of the virus.

Three experiments were carried out under similar conditions, and the results

* Kelvin, Bottomley and Baird atmospheric type of vapour lamp.

† Cernovodeanu and Henri (1910) have shown that egg albumin, gelatine and serum absorb rays of wave-lengths below 2900 Å.U.

were the same in each case. The virus of foot and mouth disease was not inactivated or apparently attenuated even after 30 minutes' exposure to radiations transmitted by ordinary glass.

In all these experiments, the virus used was one kindly sent to us by Gins and Fortner in 1925. This virus, which was isolated in 1922, gives generalized lesions on the tongue and fore-pads in nearly 100% of guinea-pigs infected by intradermal inoculation of the metatarsal pads.

The test for the absence or presence of virus in the various irradiated fluids was to inoculate samples, withdrawn from the flasks at the varying intervals, intradermally into the metatarsal pads of each of two guinea-pigs. When no primary lesions developed in the inoculated pads, the inactivation of the virus was concluded. In all cases where the virus was present, as evidenced by the development of primary vesicles on the pads of inoculated guinea-pigs, generalized lesions on the tongue and fore-pads followed later, as in controls inoculated with untreated, filtered and unfiltered lymph.

## CONCLUSIONS.

The virus of foot and mouth disease in filtrates is destroyed after 5 minutes' exposure to the radiations of the mercury vapour lamp in quartz flasks, which transmit radiations of wave-lengths from 5720–2300 Å.U.; it is inactivated after 30 minutes' exposure to the rays of the mercury-vapour lamp filtered through a sheet of vita-glass which allows the passage of rays of wave-lengths from 5720–2800 A.U. The rays of wave-lengths 5720–2300 Å.U. have no lethal action on the virus of foot and mouth disease. When the virus is suspended in unfiltered lymph or serum a protective action, due to selective absorption by the suspending fluid, was observed.

## REFERENCES.


Baker, S. L., and Peacock, P. R.—(1926) *Brit. J. Exp. Path.*, **7**, 310.

Bedson, S. P., Maitland, H. B., and Burbury, Y.—(1927) '2nd Progress Report, Foot and Mouth Disease Committee, Ministry of Agriculture,' London (H.M. Stationery Office), 70.

Cernovodeanu, P., and Henri, V.—(1910) *C.R. Acad. Sci., Paris*, **150**, 54.

Finsen, N. R., and Dreyer, G.—(1903) *Mitt. Finsens med. Lysinst.*, **3**, 72.

Illingworth, C. F. W., and Alexander, G. L.—(1927) *J. Path. Bact.*, **30**, 365.

McKinley, E. B., Fisher, R., and Holden, M.—(1926) *Proc. Soc. Exp. Biol.*, **23**, 408.

Nicolau, S., and Galloway, I. A.—(1927) *Brit. J. Exp. Path.*, **8**, 336.

Olitsky, P. K., and Gates, F. L.—(1927) *Proc. Soc. Exp. Biol.*, **24**, 431.

Rous, P.—(1913) *J. Exp. Med.*, **18**, 416.

Takaya, K.—(1926) Abstracted in *J. Amer. Med. Ass.*, 1927, **88**, 131.

# COLONY VARIANTS IN HÆMOLYTIC STREPTOCOCCI.

G. HARDY EAGLES.

*From the Bacteriological Department, Lister Institute, London.*

Received for publication November 2nd, 1928.

COWAN (1922), in the course of an attempt to explain the difficulties involved in agglutination technique as applied to streptococci, found that individual strains could be dissociated into "rough" and "smooth" types. These were strikingly different with regard to colony appearance and virulence for laboratory animals. The highly granular growth of the rough variant in broth cultures led her to conclude that it was this property which was responsible for spontaneous agglutination. Other workers, recently, have investigated the phenomenon with conflicting results. Griffith (1927) has described different types of colonies met with in scarlet fever strains but has been unable to identify rough and smooth colonies analogous to those occurring in pneumococcus cultures. Todd (1927) described two types of colonies in hæmolytic streptococci; "matt" colonies with central eminences, clearly defined margins and growing granularly in broth culture and "glossy" colonies with well-defined edges and growing with even turbidity in broth. In addition, an intermediate type with "matt" surface and no central eminence was seen frequently. The "matt" type possessed considerably greater virulence for mice than the "glossy" type. They were not, in his opinion, identical with the rough and smooth colonies described by Cowan. Andrewes (1928) described "R" and "S" colonies isolated from single strains but considered it doubtful whether these were identical with Cowan's colonies since his findings regarding virulence were exactly opposite to hers. Striking differences were found in agglutination experiments with his types, suggesting that the "smooth" forms of different strains are alike serologically while the "rough" show considerable specificity.

This finding of Andrewes seemed of sufficient importance to warrant further study of these variant colonies particularly in view of the findings of previous investigators.

Two strains were studied, a toxin-producing strain from erysipelas, which had given trouble previously by spontaneous agglutination, and the Aronson (1903) strain, obtained through the courtesy of Prof. Hahn, Hygienisches Institut, Berlin, and selected on account of its reputed high virulence for mice.

## EXPERIMENTAL WORK.

The strains from stock were grown in 10% horse serum broth over night and the resulting uniformly-turbid growth sown on a 10% serum agar plate. After overnight incubation, examination by transmitted light with the naked eye,

showed all the colonies to have a bluish colour. By low power of the microscope they appeared slightly dome-shaped, with clear-cut margins and had an even, finely granular, surface. Such an appearance has been found to be common among strains kept for some time on artificial media.

### *Method of Obtaining "Rough" Colonies.*

The method outlined above with selective subculture of colonies from serum agar to serum broth through many generations has been found satisfactory. After a number of generations, varying according to the strain employed, certain colonies have developed quite definite appearances representing a gradual change from the slightly granular colony of the stock culture to the markedly granular or pure "rough" colony. The types of colony marking this change are given in detail since it seems possible that it may be these intermediate colonies which have been described by some workers rather than the fully developed "rough" and "smooth" types. These are described in the order in which they appeared. A plate microscope has been found of great assistance in selecting colonies for subculture.

*Type I. The papillary colony.*—This colony is larger and moister than the stock colony. With the naked eye by reflected light it is bluish, with a clean-cut margin and a somewhat raised central eminence surrounded by a zone of varying size. This eminence and zone are duller and more opaque than the remainder of the colony.

Under low power of the microscope the eminence and its zone are more coarsely granular than the remainder of the colony.

Fig. 1 shows such a colony, the central eminence being darker with a lighter zone which merges into the peripheral portion.

Selective subculture of such a colony from serum broth to serum agar results, after a few generations, in a larger proportion of such colonies, some of which present the characteristic appearance to a greater degree. One of these is picked and subculture continued; always selecting those colonies showing the most marked change, until the second type occurs.

*Type II. The "wrinkled" colony.*—To the naked eye these are larger than the previous type, apt to be spreading in character and show, by reflected light, heaped-up convolutions or "wrinkles" which appear dull and rather opaque in contrast to the bluish hollows and edge of the colony.

Under low power the curious effect of a relief map is presented, the elevated portions being more coarsely granular than the hollows and margin, which is sharply defined.

Fig. 2 shows a portion of a colony of this type. The more granular ridges appear darker than the remainder.

In serum broth this type tends to a mucoid growth on the bottom of the tube.

After several generations the convolutions disappear and many colonies show a coarsely-granular mass within a finely-granular portion, as shown in the drawing (Fig. 3); inspection shows that these are single colonies rather than a fusion of two individuals. Such colonies are replaced by a third type.

*Type III. The "opaque" colony.*—These colonies are readily recognized with the naked eye by reflected light since they are smaller than the others in the same plate and are dull white.

Under low power they are coarsely granular, intermixed with fine granularity. The edge is no longer clearly defined but is woolly, with a tendency to thread-formations in loops.

In serum broth they grow in a mass on the bottom leaving a clear supernatant. On shaking, this mass breaks up into definite granules. Such a colony is shown in Fig. 4.

*Type IV. The "rough" colony.*—This final stage from stock colony to one which represents the most marked degree of coarse granularity obtained, is reached by frequent subculture of Type III colonies. Further subculturing of individuals does not bring about greater degrees of coarse granularity and all colonies on a plate possess the characteristics of the type to an equal degree. This feature has been used in this study as an evidence of purity of type.

By reflected light the colonies appear to the naked eye small and dead white with a dull surface.

Under low power a quite typical and rather striking appearance is presented. The granularity is coarse to such a degree as to give the impression of individual cocci in much-tangled long chains. The edge is completely irregular and wavy so that the whole colony resembles somewhat that of *B. anthracis.*

That the size of the colony has no relation to the degree of granularity is shown in Fig. 5, where smaller colonies are included with larger ones.

In serum broth the growth is on the bottom in coarse granular masses which cannot be dispersed on shaking.

Such colonies have been kept in serum broth in the cold for nine months. Upon subculture to broth or agar, with or without the addition of horse-serum, the cultures have been viable and have produced only type-true colonies.

*"Smooth" colonies.*—For purposes of description it has seemed advisable to describe in sequence those variant colonies derived from the stock strain which show a progressive degree of "roughness" or coarse granularity without introducing the "smooth" or finely granular type. Ordinarily, beginning with the stock colonies, search is made for these at the same time, selecting for subculture such colonies as show finer granularity than the stock colony. The method of culture is identical, and is carried on until the greatest degree of fine granularity possible has been obtained and successive subculture yields only such colonies. Such colonies are considered to be a pure strain of

---

DESCRIPTION OF PLATE.

FIG. 1. Type 1, papillary colony.
FIG. 2. Type 2, wrinked colony.
FIG. 3. Drawing, mixed rough and smooth colony.
FIG. 4. Type 3, opaque colony.
FIG. 5. Type 4, pure rough colony.
FIG. 6. Type 5, pure smooth colony.

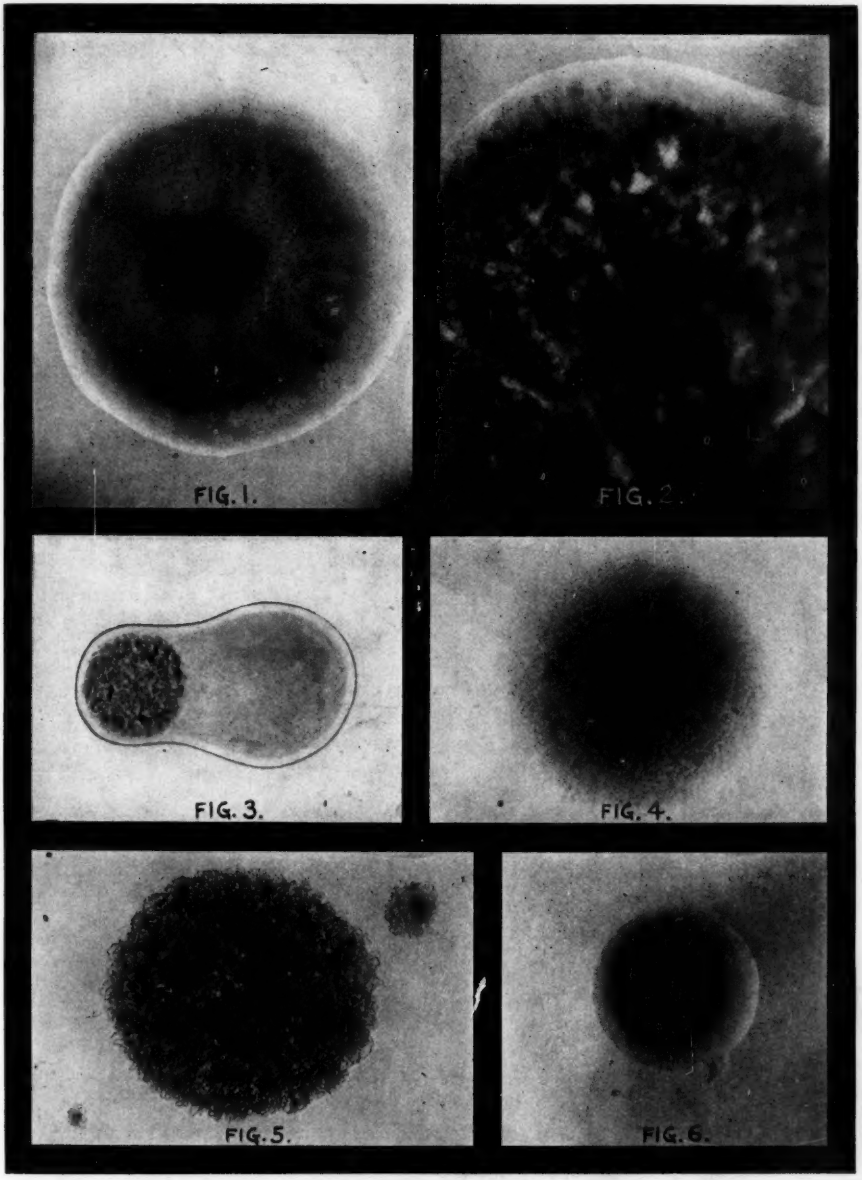


FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

Eagles.

"smooth" variant of the stock strain. In selecting colonies for subculture, therefore, it becomes necessary to recognize slight differences in granularity, a distinction which becomes increasingly difficult as selection progresses.

Such a colony is shown in Fig. 6.

"Smooth" colonies to the naked eye are rather transparent, bluish in colour and have a glistening surface. Under low power they show an evenly-dispersed, very fine granularity. The margin is clearly defined. In serum broth there is an even turbidity.

### *Characters of "Roughs" and "Smooths."*

In addition to the colony appearance and the growth in broth culture certain other characteristics have been observed.

(a) *Length of chain.*—The "smooth" type grows in short chains of a few cocci, either in fluid or on solid media. "Roughs," on the contrary, grow in exceedingly long tortuous chains, both in broth or on solid media.

(b) *Hæmolysins.*—No difference could be detected in the ability of either type to hæmolyse human or sheep's corpuscles.

(c) *Toxin production.*—Toxic filtrates from "rough" and "smooth" variants of the New York erysipelas strain were prepared and tested in known Dick-positive volunteers. No difference could be detected in size of reaction of either type when compared with toxin prepared from the stock strain. A satisfactory toxin from the Aronson strain was not obtained.

(d) *Saline agglutinability.*—Saline was prepared in strengths ranging from 0·85% to 0·1%. Growth from rough and smooth types and the "wrinkled" and "opaque" intermediate forms was removed from solid media with distilled water, emulsified by capillary pipette and made up with distilled water to apparently equal density. Drops of suspension and saline were placed side by side on a glass slide and mixed with a loop while being observed under low power.

"Rough" growth was agglutinated by 0·1% saline.
"Opaque" by 0·2% saline.
"Wrinkled" by 0·4% saline.
"Smooth" by 0·4 to 0·5% saline.

(e) *Virulence for mice.*—Injections were made intraperitoneally of "rough" and "smooth" variants and from the "wrinkled" and "opaque" intermediate forms in 0·1 c.c. amounts of falling dilutions of plain broth cultures. Animals were examined daily and deaths recorded up to 14 days after inoculation.

In order to make comparison as close as possible and to avoid injecting granular masses, all cultures were broken up by forcing through a capillary pipette and density made as nearly equal as possible. Serum was not used for enrichment, in order to avoid injecting a substance which might accelerate growth intraperitoneally.

Table I shows the comparative virulence of the four types of colonies derived from the New York erysipelas strain. Table II shows the same four types from the Aronson strain.

TABLE I.

| Dose in c.c.'s | Deaths in days. N.Y. "R." | N.Y. "O." | N.Y. "W." | N.Y. "S." |
|---|---|---|---|---|
| 0·1 | S | S | S | S |
| 0·2 | S | S | 3 days | 3 days. |
| 0·3 | S | S | 2 ,, | 2 ,, |
| 0·4 | S | S | 1 day | 2 ,, |
| 0·5 | S | S | 1 ,, | 1 day |
| 0·6 | 1 day | S | 1 ,, | 1 ,, |
| 0·7 | 1 ,, | 3 days | 1 ,, | 1 ,, |
| 0·8 | 1 ,, | 3 ,, | 1 ,, | 1 ,, |
| 0·9 | 1 ,, | 2 ,, | 1 ,, | 1 ,, |
| 1·0 | 1 ,, | 2 ,, | 1 ,, | 1 ,, |

N.Y. "R" = New York strain "rough" variant.
N.Y. "O" = ,, ,, "opaque" variant.
N.Y. "W" = ,, ,, "wrinkled" variant.
N.Y. "S" = ,, ,, "smooth" variant.
S = Survivals.

TABLE II.

| Dose in c.c.'s | Deaths in days. A "R." | A "O." | A "W." | A "S." |
|---|---|---|---|---|
| 0·1 | 1 day | 2 days | 4 days | 4 days. |
| 0·02 | 2 days | 2 ,, | 4 ,, | 4 ,, |
| 0·01 | 2 ,, | 3 ,, | 3 ,, | S |
| 0·001 | 2 ,, | 3 ,, | 3 ,, | S |
| 0·0001 | 2 ,, | 3 ,, | S | S |
| 0·00001 | 2 ,, | 4 ,, | S | S |
| 0·000001 | 3 ,, | 5 ,, | S | S |
| 0·0000001 | 4 ,, | S | S | S |

A "R" = Aronson strain "rough" variant.
A "O" = ,, ,, "opaque" variant.
A "W" = ,, ,, "wrinkled" variant.
A "S" = ,, ,, "smooth" variant.
S = Survivals.

## DISCUSSION.

"Rough" and "smooth" variant colonies apparently similar to those described by Cowan, have been obtained by selective subculture from individual colonies of stock strains of hæmolytic streptococci.

The development from stock colony to the coarsely granular rough variant has resulted in a replacement of the slight granularity of the former by increasingly coarser granularity in such a manner that three well-defined intermediate variants have been recognized. These intermediate forms are important in that they may lead to confusion when pure rough strains are desired. Colonies with papillæ and "matt" appearance described by Todd and the second and third types from scarlet fever described by Griffith bear

considerable resemblance to the "papillary" and "wrinkled" colonies of this study. The "opaque" type would seem to be quite similar to the rough forms described by Andrewes. "Smooth" strains show a gradual replacement of the inherent fine granularity of the stock colony by a still finer granularity. For a clear understanding of pure rough and smooth forms in streptococci, therefore, it is necessary to recognize that gradation forms exist and that pure variants are developed only after long selective subculture. These pure forms unlike the intermediate variants, which are unstable and readily change their appearance, breed true to type on successive subculture. This may be explained by supposing the stock colony to possess a mixture of coarse and fine granularity. Selective subculture results in the "rough," by breeding out the finely granular element and in the "smooth," the coarsely granular. The intermediate forms, possessing a mixture of the two in varying proportion, readily alter their appearance according to which factor predominates.

As pointed out by Andrewes the ability of a strain to form a good suspension depends largely on the amount of "smoothness" possessed by its colonies, from the present work there is good evidence that both properties are probably dependent on the length of chain. The point seems worthy of consideration when one remembers that some workers depend on rapid subculture to convert on auto-agglutinable, freshly-isolated strain to one forming a stable suspension. This would seem to result in producing in the strains a preponderance of shorter chains as is found in many stock and in smooth colonies. That this may lead to serious pitfalls in interpretation of agglutination results has been definitely shown by Andrewes.

The question of virulence of these types for mice is confusing. As set out in Table I the pure "R" and "S" variants agree with Cowan's findings in that the "S" form is more virulent than the "R." In the Aronson strain (Table II) the exact opposite is the case where the "R" form is highly virulent while the "S" is of relatively low virulence. In the intermediate forms there seems to be a definite relationship between virulence and preponderance of coarse over fine granularity in the colony. This is shown clearly in that the "opaque" form of Aronson strain, which contains much more coarsely-granular element than the "wrinkled," is more virulent than either the "W" or "S" forms which have a preponderance of "smooth" or finely granular element.

The explanation of this lack of conformity in virulence in the "R" and "S" forms in the two strains under study is not clear. It may be that the Aronson strain, possessing as it does extraordinarily high virulence for mice, is in some unknown way different from other strains and constitutes an exception to the general rule. The presence of serum in the media appears to exert considerable influence on the virulence of the "R" form. It has been found that frequent subculture of the "R" variant in serum broth maintains its virulence, though when it is stored on serum agar in the cold its virulence is practically lost in a few weeks, though the roughness persists. It has been found possible to restore the virulence by frequent subculture in serum incorporated either in broth or agar.

When cultures of "rough" variant in which a trace of "S" persists are subcultured frequently in serum-containing media reversal to the stock type of colony takes place, the intermediate types described in this study again making

their appearance. In such it has been noted that virulence falls as the culture loses the "rough" element.

It has been suggested to me that the Aronson "R" form owes its colony appearance and enhanced virulence to an excessive amount of lipoid substance derived from the serum in the medium in which it is grown. From our observations this would seem probable were it not that extraction of the lipoid by methods described by Bruce White (1928) resulted in an apparently equal amount of material from both "R" and "S" variants.

While it is not desirable to formulate any definite conclusions from a study based on the behaviour of two strains it would seem that colony appearance is not a reliable index of virulence but that other factors must be considered.

## CONCLUSIONS.

"Rough" or very coarsely granular colonies and "smooth" or very finely granular colonies, apparently similar morphologically to those described by Cowan, have been identified by selective subculture of individual stock strains of hæmolytic streptococci.

Intermediate variants have also been described.

Colony appearance is not a reliable indication of virulence in hæmolytic streptococci in as much as the property of enhanced virulence in one strain to the "rough" and in the other to the "smooth" variety.

In a toxin-producing strain "rough" and "smooth" variants have been found to produce apparently equal amounts of toxin.

## REFERENCES.

ANDREWES, F. W.—(1928) Abst. Proc. Path. Soc. Gt. Brit. and Ireland, *J. Path. Bact.*, **31**, 132.

COWAN, MARY L.—(1922) *Brit. J. Exp. Path.*, **3**, 187.

GRIFFITH, F.—(1927) *J. Hyg.*, **26**, 4, 363.

TODD, E. W.—(1927) *Brit. J. Exp. Path.*, **8**, 289.

WHITE, P. B.—(1928) *J. Path. Bact.*, **31**, 423.

# ON THE PHAGOCYTOSIS OF HÆMOLYTIC STREPTOCOCCI OF HIGH AND LOW VIRULENCE BY THE BLOOD OF PATIENTS INFECTED WITH THAT ORGANISM.*

RONALD HARE, M.B., B.S.LOND.†

*From the Institute of Pathology and Research, St. Mary's Hospital, London.*

Received for publication November 15th, 1928.

TODD (1927[1]) has described recently a method by which the rate of increase or decrease of hæmolytic streptococci in defibrinated blood may be measured. For this purpose he employs a mixing machine on which the blood and microbes are rotated to prevent "settling" of the cells during incubation.

This method seemed likely to provide a means whereby the bactericidal power of the blood of patients infected with that organism might be measured with accuracy, and with this object tests have been performed on the blood of a number of cases of puerperal septicæmia. These experiments at first gave incomprehensible results until it was found that the virulence of the test organism was of great importance.

But it is first necessary to refer to Todd's (1927[2]) further work on virulence, in which he has shown that strains of hæmolytic streptococci which are virulent for mice are able to multiply freely in normal human defibrinated blood. Conversely, strains which are avirulent for mice are killed by normal human blood. In so doing, he has correlated the virulence of these streptococci for mice with ability to survive in human blood. And in consequence of this work, the term "virulent" in this paper connotes ability to survive and multiply in normal human defibrinated blood.

In the bactericidal experiments with the blood of the infected patients, virulent strains were found to multiply much less readily in their blood than in the blood of normal persons. But when avirulent strains were introduced into the blood the converse took place. Such strains may be killed less easily in the blood of patients than in the blood of normal persons. They may even multiply in such blood.

In seeking an explanation for this apparent anomaly, I regularly found that the phagocytic power of the polymorphonuclear leucocytes in the blood of such patients is, broadly speaking, what might have been predicted from the growth tests. That is to say, the phagocytic power of the blood for virulent streptococci may be higher than the normal, but lower when avirulent strains are employed. Typical examples of this are given in Tables I and II, and the probable reasons for this variation are subsequently studied in detail.

* This paper formed part of the essay which was awarded the Katherine Bishop Harman Prize of the British Medical Association in 1928.

† Working with a grant from the Medical Research Council.

*Source and Cultivation of the Streptococcal Cultures Employed.*

Strains of hæmolytic streptococci isolated from a septic focus or the blood of cases of puerperal fever were alone used.

Virulent strains were produced by continued subcultivation in normal human serum, and avirulent strains by cultivation on agar or (in one instance) in broth (Todd, 1927[2]).

For the purposes of the experiments which follow, subcultures from strains prepared as above were made in 5% hydrocele fluid broth and incubated for 16 hours.

The experiments were carried out with the defibrinated blood of puerperal fever patients under treatment in either the hospitals of the Metropolitan Asylums Board or the Hammersmith Hospital. All cases were infected with hæmolytic streptococci as judged by blood and uterine culture.

The phagocytic experiments herein described were all carried out by a method which involves the mixing of blood and microbes on a Todd's mixing machine during the period of incubation.

PHAGOCYTIC TESTS WITH STREPTOCOCCI OF HIGH AND LOW VIRULENCE.

In the first experiment the relative phagocytic powers of the polymorphonuclear leucocytes of a patient suffering from severe streptococcal septicæmia and of a normal control are measured with streptococci of different virulence.

*Experiment 1.*

*Bloods investigated.*—(1) Mrs. W—, puerperal septicæmia (1 c.c. of blood grew 1000 colonies of hæmolytic streptococci). (2) Normal R.H. for comparison.

*Method.*—2·5 c.mm. of the appropriate cultures were placed in capsules. To each, 50 c.mm. of defibrinated blood was added, and after sealing, the tubes were incubated on the mixing machine for 15 minutes. They were then opened and opsonic films made. After staining, the number of cocci in 100 polymorphonuclears in each case was counted (Table I).

TABLE I.

| Description of culture. | Number of cocci in 100 polymorphs in— | | Phagocytic index. |
|---|---|---|---|
| | Patient's blood. | Normal blood. | |
| *Virulent:* 16-hour broth culture of Edwardson previously subcultured 40 times in normal serum | 736 | 190 | $\frac{736}{190} = 3{\cdot}8$ |
| *Avirulent:* 16-hour broth culture of patient's own blood streptococcus attenuated by growth in broth | 576 | 570 | $\frac{576}{570} = 1$ |

*Virulence test of the cultures employed in the above experiment.*—Mixtures, as above, of normal defibrinated blood and dilutions of the streptococci were incubated on the mixing machine for 1 hour (Todd's method), and samples were then explanted in melted blood-agar.

*Result.*—The virulent cocci multiplied during the hour from 73 to 3000, and the avirulent diminished from 38 to 0.

I have carried out a number of similar estimations with the blood of other patients. Some of these are given in Table II. In each instance the number of cocci phagocyted by 100 polymorphs is given, that by the patient forming the numerator and that by the normal the denominator of the fraction. The phagocytic indices calculated from these figures are also included.

I have also determined the phagocytic indices with staphylococci, and these are given in the third column. This microbe was chosen because it gives easily counted films, and the patients were, so far as is known, not infected with it.

The results of concurrent tests made for the determination of the virulence of the microbes are omitted from the table for the sake of brevity. They were carried out in many instances with results similar to those given under Experiment 1.

In the majority of instances the two strains of streptococci employed to test the blood of one individual were derived from the same parent strain. The homologous microbes were not usually available at the time of the experiments.

The cocci phagocyted by the polymorphonuclear leucocytes are alone considered. Those taken up by the large mononuclears form but a small proportion of those ingested.

TABLE II.

| Name of patient. | Virulent culture of streptococci. | Avirulent culture of streptococci. | Culture of staphylococcus. |
|---|---|---|---|
| E— . . . | $\frac{107}{12} = 8{\cdot}9$ | $\frac{326}{1600} = 0{\cdot}2$ | $\frac{793}{969} = 0{\cdot}8$ |
| M— . . . | $\frac{312}{79} = 3{\cdot}9$ | $\frac{590}{900} = 0{\cdot}6$ | — |
| G— . . . | $\frac{407}{101} = 4{\cdot}0$ | $\frac{872}{710} = 1{\cdot}1$ | $\frac{367}{561} = 0{\cdot}6$ |
| J— . . . | $\frac{285}{169} = 1{\cdot}6$ | $\frac{731}{1041} = 0{\cdot}7$ | $\frac{436}{736} = 0{\cdot}5$ |
| S— . . . | $\frac{895}{281} = 3{\cdot}1$ | $\frac{592}{754} = 0{\cdot}7$ | $\frac{570}{578} = 0{\cdot}9$ |
| B— . . . | $\frac{417}{247} = 1{\cdot}6$ | $\frac{890}{679} = 1{\cdot}3$ | $\frac{270}{184} = 1{\cdot}4$ |
| Q— . . . | $\frac{125}{37} = 3{\cdot}3$ | $\frac{109}{469} = 0{\cdot}2$ | — |
| N— . . . | $\frac{892}{168} = 5{\cdot}3$ | $\frac{562}{923} = 0{\cdot}6$ | — |

The patients named in this table were all severely ill, five out of the eight having a hæmolytic streptococcal septicæmia. The same type of result as that given in Experiment 1 is seen. The phagocytic index is high with virulent microbes; with those of low virulence it tends to be low; while with a heterologous microbe such as staphylococcus it is usually below 1.*

## FURTHER ANALYSIS OF THE RESULTS OBTAINED WITH DEFIBRINATED BLOOD.

The indices obtained in the last section represent the relative behaviour of each set of corpuscles in the homologous serum. Therefore changes in both serum and cells may affect the results obtained.

Shattock and Dudgeon (1908) showed that in a number of diseases, the number of microbes taken up by the patients' leucocytes might be much below that taken up by normal leucocytes. Wright, Colebrook and Storer (1923) and Colebrook (1925) demonstrated that in septic infections the efficiency of the leucocytes was definitely subnormal when tested in normal serum.

Thus when defibrinated blood is examined, the results depend upon two quite distinct factors—on the opsonization of the microbes, and on the phagocytic abilities of the leucocytes. In order to analyse the broad result obtained with the blood, the relative importance of these two factors must be examined separately. This is done conveniently by the "chiastic procedure" of Wright, Colebrook and Storer (1923).

In Experiments 2 and 3 which follow, the phagocytic estimations which constitute this are carried out with streptococci of high and low virulence.

*Experiment 2.*

*Bloods investigated.*—1. Mrs. N—. (Puerperal septicæmia.) 2. R. M. F. Normal blood.

*Method.*—Both bloods were centrifuged and the sera removed; each set of corpuscles was washed in physiological salt solution three times. 25 c.mm. of the cells and 25 c.mm. of the sera, in mixtures as indicated below, were added to 2·5 c.mm. of virulent or avirulent cultures of a hæmolytic streptococcus (Jonsson†). They were incubated on the mixing machine for 15 minutes when opsonic films were made. The number of cocci per 100 polymorphonuclear leucocytes was then counted (Table III).

* In several instances the appearance of the leucocytes in the stained films showed evidence of extreme degeneration. A proportion were merely formless masses of pale staining protoplasm quite unrecognizable as any particular type of cell. These do not phagocyte microbes at all. In the process of counting they are necessarily neglected, and in so doing there is perforce introduced a "selection" of cells. Thus a somewhat spurious value may possibly be given to the phagocytic counts with the cells of the patient. This has occurred seldom, but even in these instances, be it noted, the change in the index with virulent and avirulent microbes is not affected.

† The virulent strain had been subcultured 35 times in normal human serum while the avirulent strain had undergone 63 subcultures on plain agar.

TABLE III.

| Mixtures. | Virulent culture. | Avirulent culture. |
|---|---|---|
| (1) In a mixture of normal cells + normal serum 100 polymorphs contain— | 183 cocci | 645 cocci |
| (2) In a mixture of normal cells + patients' serum 100 polymorphs contain— | 1390 „ | 789 „ |
| (3) In a mixture of patients' cells + patients' serum 100 polymorphs contain— | 1039 „ | 410 „ |
| (4) In a mixture of patients' cells + normal serum 100 polymorphs contain— | 118 „ | 435 „ |
| These figures give— | | |
| Opsonic index — Mixture (2) / Mixture (1) | $\frac{1390}{183} = 7{\cdot}5$ | $\frac{789}{645} = 1{\cdot}2$ |
| Phagocytic efficiency of the leucocytes operating in normal serum — Mixture (4) / Mixture (1) | $\frac{118}{183} = 0{\cdot}6$ | $\frac{435}{645} = 0{\cdot}6$ |
| Phagocytic efficiency of the leucocytes and serum combined (operating in their own serum) — Mixture (3) / Mixture (1) | $\frac{1039}{183} = 5$ | $\frac{416}{645} = 0{\cdot}6$ |

In the next experiment exactly the same procedures were employed, but in addition, the virulence or power of the streptococci to multiply in human blood is indicated more precisely.

*Experiment 3.*

*Bloods investigated.*—1. Mrs. M—. (Severe puerperal septicæmia.) 2. R. H—. Normal.

*Method.*—A chiastic test was carried out as in the last experiment. The rate of growth of the streptococci in normal blood was ascertained by the same method as that described in Experiment 1. The result is shown in Table IV.

TABLE IV.

| *Chiastic test:* | Virulent culture. | Avirulent culture. |
|---|---|---|
| Opsonic index | $\frac{342}{80} = 4{\cdot}2$ | $\frac{985}{826} = 1{\cdot}1$ |
| Phagocytic efficiency of the leucocytes of the patient operating in normal serum | $\frac{47}{80} = 0{\cdot}58$ | $\frac{589}{826} = 0{\cdot}71$ |
| Phagocytic efficiency of the leucocytes and serum combined (operating in their native serum) | $\frac{171}{80} = 2{\cdot}1$ | $\frac{733}{826} = 0{\cdot}88$ |
| *Virulence test:* | | |
| (Comparison between the number of microbes added and those present after one hour's incubation in normal blood.) | | |
| Number of colonies grown in explants before incubation | 176 | 1216 |
| Number of colonies grown in explants after incubation for 1 hour in normal blood | 1218 | 742 |

*Comment on the Results Obtained in the Previous Experiments.*

In these estimations the term " phagocytic efficiency of the cells and serum combined " is an index, which is in every way comparable to that known as the phagocytic index of the defibrinated blood, seeing that it is derived from an examination of the phagocytic power of each set of leucocytes in the homologous serum. The same type of result as that given by defibrinated blood set out in Tables I and II is obtained, namely a high index with virulent strains and one near unity with avirulent strains.

The other indices analyse this more precisely.

That which indicates the efficiency of the leucocytes is the same with both strains of streptococci, showing that these cells do not distinguish between virulent and avirulent strains when suspended in normal serum. This index, albeit, is low, and shows that their efficiency is subnormal.

But the opsonic index is very variable, being high with virulent and low with avirulent strains.

In the patient's blood the serum is able, in virtue of its high opsonizing power for virulent strains, to compensate for the deficiencies of the leucocytes to such good purpose that the indices obtained with such microbes are high. But tested with avirulent strains, the opsonizing power of the patients' serum is apparently no better than the normal. With such a culture, therefore, the total phagocytic efficiency of the defibrinated blood is below normal.

These experiments all show that when dealing with defibrinated blood and virulent streptococci, the opsonizing power of the serum dominates the picture, giving a high phagocytic index. But when organisms of low virulence are employed this does not occur; the opsonizing power of both normal and patients' serum is about equal. The outcome of this is that the diminished phagocytic activities of the leucocytes are reflected in the counts, and a low index is the result.

Substantially the same remarks can be made about the phagocytic indices obtained with staphylococci, where the opsonizing powers of the sera are about equal, and the diminished activity of the leucocytes is mainly responsible for the low indices obtained.

It was not, at first, at all clear why the opsonic index should vary with the virulence of the microbes employed in its estimation. But the idea was put forward as a hypothesis that practically all the avirulent microbes were phagocyted in the presence of both sera. In such circumstances an index very far removed from unity is a practical impossibility. But a large proportion of the virulent microbes might resist phagocytosis by the cells of normal blood, and in consequence the presence of opsonins in another blood able to opsonize such microbes would be detected. To determine whether this hypothesis was correct the following experiment was undertaken:

A phagocytic mixture was set up employing normal defibrinated blood and virulent and avirulent microbes. The total number of microbes implanted was ascertained and also the total number phagocyted. A comparison was then made between the total number phagocyted and the number incorporated in the mixture.

*Experiment 4.*

Virulent and avirulent cultures were employed.

*Method.*—2·5 c.mm. of each culture was added to 50 c.mm. of normal defibrinated blood. They were incubated on the mixing machine for 15 minutes when opsonic films were made. The total number of cocci was ascertained in each instance by a method of counting described by Sir Almroth Wright (Wright and Colebrook, 1921).

The total number of cocci phagocyted by the blood was also found from a consideration of (1) the number of cocci ingested by 100 leucocytes, (2) the white cell, and (3) the differential count.

*Avirulent Culture :*

| | |
|---|---|
| Total number of cocci added to 50 c.mm. of blood . . | 1,004,152 |
| Average number of cocci phagocyted by each polymorph . | 4·23 |
| White cell count per 1 c.mm. of the blood employed . . | 6212 |
| Differential count gave 59% polymorphs. | |
| Total number of cocci phagocyted by the polymorphs of the 50 c.mm. of blood . . . . . | 774,936 |

Thus 77% of the added microbes were phagocyted in the 15 minutes of mixing.*

*Virulent Culture :*

| | |
|---|---|
| Total number of cocci added to the 50 c.mm. of blood . | 1,025,000 |
| Average number of cocci phagocyted by each polymorph . | 0·33 |
| Total number of cocci phagocyted by the polymorphs of the 50 c.mm. of blood . . . . . | 60,556 |

Thus 5% of the added microbes were phagocyted in the 15 minutes of mixing.

*Comment (1) on the above experiment.*—This experiment shows that normal serum is quite adequate for its purpose, and can secure the phagocytosis of almost the whole of the added avirulent microbes in 15 minutes. They are therefore fully opsonized by the normal serum alone. More phagocytosis when the normal is replaced by one containing more opsonin (that of a patient for instance) can hardly be expected.

This applies to implants which have been used throughout this work—that is to say ones which give counts in the neighbourhood of 1 to 10 per cell. The suggestion has been made that bigger implants might enable one to detect differences between two sera even with avirulent microbes. It was found, however, that even when the counts reached nearly 50 per cell no difference could be detected between the two sera.

*Comment (2) on the above experiment.*—Normal serum is inadequate for the opsonization of the virulent microbes. This is shown by the fact that there is, when normal serum is compared with serum from a patient containing

* If the organisms in the large mononuclears be taken into account the percentage phagocyted reaches approximately 97, showing that almost the whole of the implant had been phagocyted in the 15 minutes of mixing.

substances able to opsonize virulent microbes, an obvious difference in their performances. This difference is expressed in the form of a high opsonic index.

## FURTHER EXAMINATION OF THE OPSONINS PRESENT IN THE SERUM OF PATIENTS.

*Section A: On the Passive Transference of Opsonins for Virulent Streptococci to Normal Blood.*

In view of the possibility of the use of sera containing opsonins for virulent streptococci in serum therapy or in blood transfusion, it was thought to be highly desirable to ascertain whether this power of the serum could be transferred passively to normal human blood. With this in view, experiments have been performed in which small quantities of such sera were incorporated in normal defibrinated blood to which virulent organisms are then added. Typical results are given in Experiments 5 and 6, and show that such a proceeding does lead to increased phagocytosis of virulent organisms.

*Experiment 5.*

50 c.mm. of fresh normal defibrinated blood were placed in each of several capsules. To each, 5 c.mm. of serum from a case of septicæmia (J—) or a normal control and 2·5 c.mm. of streptococci in broth culture were added. The tubes were incubated on the mixing machine for 15 minutes when opsonic films were made.

This was performed with three different virulent strains of streptococci, and to make the experiment more instructive, with the avirulent strains of the same streptococci (Table V).

TABLE V.

| | Number of cocci in 100 polymorphs. | |
|---|---|---|
| | In blood + patient's serum. | In blood + normal serum. |
| Culture 1.—H— (virulent) | 910 | 442 |
| ,, 2.—E— ,, | 947 | 389 |
| ,, 3.—J— ,, | 957 | 267 |
| ,, 4.—H— (avirulent) | 29 | 73 |
| ,, 5.—E— ,, | 437 | 381 |
| ,, 6.—J— ,, | 952 | 856 |

It is quite evident from these results that when a small quantity of patients' serum is present in normal blood there occurs much increased phagocytosis of virulent strains. But, as might be expected, there is little or no alteration when avirulent strains are employed.

In the next experiment one virulent culture is employed and several specimens of serum from infected patients are tested.

*Experiment* 6.

Culture was a virulent strain of E— hæmolytic streptococcus. Method as in the last experiment. The result is shown in Table VI.

TABLE VI.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| In normal blood | + | normal serum | (A.F.H.) | . | . | . | . | 50 polymorphs contain | 258 | cocci. |
| „ | + | „ | (R.H.) | . | . | . | . | „ | 341 | „ |
| „ | + | serum of infected patient | (H—) | . | . | . | . | „ | 570 | „ |
| „ | + | „ | „ | (G—) | . | . | . | „ | 757 | „ |
| „ | + | „ | „ | (E—) | . | . | . | „ | 684 | „ |
| „ | + | „ | „ | (M—) | . | . | . | „ | 592 | „ |

In a subsequent experiment in a later section it will be shown that the presence of so small a concentration as 1 in 1,000,000 of such serum in normal defibrinated blood leads to markedly increased phagocytosis of the virulent organisms.

No indications of group specificity in this reaction have been obtained—that is to say, all hæmolytic streptococci I have tested, provided they be of adequate virulence, will serve to demonstrate this property in the serum. It is not by any means confined to the homologous organisms.

*Section B : On the Effect of Heat on the High Opsonizing Power of the Serum of Infected Persons for Virulent Organisms.*

Neufeld and Rimpau (1904) showed that the phagocytosis of virulent organisms was facilitated by substances present in the serum of immunized animals; they also showed that the substances in question (bacteriotropins) resisted 55° to 60° C. for a short time. The serum of infected human beings having a similar action in promoting the phagocytosis of virulent organisms, it is demonstrated in the next experiment that the power to do so is not destroyed by heating to 55° C. for 30 minutes.

*Experiment* 7.

Samples of the sera of patients (J—, H— and N—) and of a normal were heated at 55° C. for 30 minutes. Phagocytic experiments similar to the last were set up with each specimen of serum, both heated and unheated. A virulent streptococcus was employed (Table VII).

TABLE VII.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 100 polymorphs | in normal blood | + | J— | serum | unheated | contain | 997 | cocci. |
| „ | „ | + | „ | „ | heated | „ | 939 | „ |
| „ | „ | + | H— | „ | unheated | „ | 827 | „ |
| „ | „ | + | „ | „ | heated | „ | 1125 | „ |
| „ | „ | + | N— | „ | unheated | „ | 817 | „ |
| „ | „ | + | „ | „ | heated | „ | 692 | „ |
| „ | „ | + | normal | „ | unheated | „ | 362 | „ |
| „ | „ | + | „ | „ | heated | „ | 360 | „ |

The last experiment shows quite definitely that patients heavily infected with hæmolytic streptococci possess in their serum a substance capable of opsonizing virulent streptococci which is not destroyed by heat. There is no, or at the most only a trifling, reduction in opsonic power after such treatment.

This extends to naturally infected human beings the work of Neufeld and Rimpau (1904), who found a similar substance in the serum of animals immunized against streptococci. Jotten (1917) found similar bodies in the sera of women who were receiving prophylactic injections of streptococcal vaccine.

But it was shown by Wright and Reid (1906), working with the serum of tuberculous patients, that the opsonic power for *B. tuberculosis* is diminished as a result of heat, although to a less degree than with normal serum. And the same holds, as shown by Cummins and Acland (1925), with the opsonins which operate upon the microbes which produce secondary infections in that disease.

There is thus a divergence between the results of these two authors and the experiments of Neufeld and myself.

It is probable that an explanation of this divergence may lie in the fact that heated serum when used as the sole suspension medium, as in the experiments of Wright and of Cummins, may to some extent paralyse the phagocytic activities of the leucocytes. And in consequence, the opsonization of the microbes cannot be satisfactorily assessed. This, as a source of fallacy, is avoided in the experiments herein described by the dilution of the heated serum in a relatively large volume of unheated normal serum.

The unheated normal serum is itself unable to opsonize virulent microbes. Thus the leucocytes are suspended in a medium which is not injurious to them, and which, at the same time, has but little opsonic power. Then, if a small volume of patient's serum be incorporated in it, the presence of an opsonin able to opsonize virulent organisms can be readily and easily detected. And owing to the dilution, such serum, when heated, does not of itself harm the leucocytes.

It will thus be seen that this work can only be satisfactorily carried out with virulent microbes, or, at all events, those which are not opsonized in normal serum.

### EXPERIMENTS SHOWING THAT THE INCREASED PHAGOCYTOSIS OF VIRULENT STREPTOCOCCI WHICH OCCURS WHEN THE SERUM OF INFECTED PATIENTS IS ADDED TO NORMAL BLOOD CAN BE CORRELATED WITH AN INCREASED BACTERICIDAL EFFECT ON THE SAME COCCI.

Todd (1927[3]) has described experiments in which the addition of small quantities of serum derived from patients with streptococcal septicæmia to normal blood has the effect of reducing the rate of multiplication of the homologous virulent streptococci in that blood. The experiments already given in this paper raise the question whether these results of Todd's may have been due to increased phagocytosis of the cocci. Todd considered this as a possible cause of the effects he was studying, but was unable to satisfy himself that increased phagocytosis accompanied them. But the sera of all the streptococcal infected patients I have examined gave increased phagocytosis

of virulent cocci when added to normal blood. And this indicated so strongly that increased phagocytosis was, at least, a possible cause of the reduction in the rate of growth of these virulent cocci that the whole question called for re-examination.

In the first experiment a small quantity of serum from a septicæmia patient is added to normal defibrinated blood. Phagocytic and growth experiments are carried out in parallel with the same virulent culture.

*Experiment 8.*

A virulent culture was employed.

(a) *Growth experiment.*—100 c.mm. of normal defibrinated blood was placed in each of several capsules. To each 5 c.mm. of serum from a patient (J—) was added (or 5 c.mm. of normal control serum), and then 10 c.mm. of a suitable dilution of the culture. These were incubated on the mixing machine for 1 hour, when 50 c.mm. of each was explanted into melted blood-agar. A control volume explanted at the start gave the number of organisms implanted in each instance. Result:

| | |
|---|---|
| Number of microbes implanted . . . . . | 864 |
| ,, survivors in normal blood + patient's serum . | 648 |
| ,, ,, ,, + normal serum . . | 2128 |

(b) *Phagocytic experiment.*—Mixtures as above of blood and the two sera were set up, and to each tube 2·5 c.mm. of the same culture (undiluted) were added. These were incubated on the mixing machine for 15 minutes when opsonic films were made. Result:

100 polymorphs in a mixture of normal blood and patient's serum contain 340 cocci.

100 ,, ,, ,, ,, normal serum contain 151 cocci.

This experiment shows that there is both increased phagocytosis and a decrease in the streptococcal population when the patient's serum is present. The probability is that the latter is due to bactericidal action by the leucocytes on the cocci. But it is evident that several factors, alone or in concert, may contribute to this apparent bactericidal action. These include agglutination, an increase in length of the chains (Pfaundler's thread reaction), alteration in the growth-rate of the cocci in the serum, or possible stimulation of the leucocytes. To ascertain whether these factors were of great importance the following experiment was devised, in which the cocci are opsonized in the sera for a short time and then washed in saline before their addition to the blood, thus removing all but traces of the serum:

*Experiment 9.*

100 c.mm. of serum from a case of septicæmia (or from a normal control) was added to 50 c.mm. of a virulent streptococcal culture. The two mixtures were incubated for 3 minutes. They were centrifuged hard, the clear supernatant fluid removed, and a large volume of saline added. After thorough washing the cocci were spun down again and resuspended in 50 c.mm. saline. These two suspensions were employed in the following experiments:

(*a*) 10 c.mm. of suitable dilutions of each suspension was added to 100 c.mm.

of normal human defibrinated blood and incubated on the mixing machine for 1 hour, at the end of which period 50 c.mm. was explanted into melted blood-agar. 50 c.mm. explanted from a control volume at the start gave the number of organisms present added in each instance (Table VIII).

TABLE VIII.

| | Culture opsonized in— | |
|---|---|---|
| | Patient's serum. | Normal serum. |
| Number of microbes implanted . . | 736 | 664 |
| Number of survivors after 1 hour's mixing in normal blood . . . . . | 806 | 1344 |

(*b*) A phagocytic experiment with each suspension was set up and the tubes mixed for 15 minutes. Result:

100 polymorphs in normal blood contain 1055 cocci opsonized previously in patient's serum.

100 „ „ „ 456 cocci opsonized previously in normal serum.

(*c*) Exactly the same mixtures were set up as under (*a*), except that normal serum replaced the defibrinated blood, the effect of the leucocytes being thereby eliminated (Table IX).

TABLE IX.

| | Culture opsonized in— | |
|---|---|---|
| | Patient's serum. | Normal serum. |
| Number of microbes implanted . . | 736 | 664 |
| Number of survivors present after mixing for 1 hour in normal serum . . . | 1367 | 1377 |

(*d*) 2·5 c.mm. of the undiluted suspensions and 100 c.mm. of fresh normal serum were put on the mixing machine in capsules with the other tubes. At the end of the period of mixing films were made, stained and examined for agglutination or chain-formation. There was no agglutination in either mixture and the chains of cocci were equal in length.

*Comment.*—There is no alteration in the vegetative characters of the organisms. The rate of growth of the two sets of microbes in serum alone is about equal; there is no tendency to chain formation or to agglutination which would simulate reduction in the rate of multiplication. But there is, quite clearly, increased phagocytosis, and at the same time reduction in the rate of multiplication when the leucocytes are present.

In the two preceding experiments the phagocytic mixtures were only incubated for 15 minutes and the mixtures in the growth experiments for 1 hour. Obviously the effects of the added serum must operate throughout the period of incubation of the growth experiment in order to give the modifications in growth which take place, and in other experiments not given here it was found that the phagocytosis of the microbes, in greater numbers when the serum of a patient was present, continued throughout the period of growth.

In the preceding experiments the patient's serum has been operating in a dilution of 1 in 10 or 20. In view of previous findings in phagocytic experiments, the effect of further dilution on both phagocytosis and bactericidal power was tested.

*Experiment 10.*

To normal defibrinated blood was added one-tenth of its volume of serial dilutions of a specimen of serum from a case of septicæmia. A control mixture was also set up with undiluted normal serum. The mixtures of serum and blood were then employed in phagocytic and growth tests with a culture of virulent streptococci.

(*a*) Growth experiment: 100 c.mm. of each mixture of blood and serum was added to 10 c.mm. of a suitable dilution of the culture and incubated on the mixing machine for one hour, when explants were made (Table X).

TABLE X.

| Concentration of serum present in the blood. | Number of survivors after 1 hour mixing. |
|---|---|
| Patient's serum 1/10 | 136 |
| ,, ,, 1/100 | 944 |
| ,, ,, 1/1000 | 992 |
| ,, ,, 1/10,000 | 1208 |
| ,, ,, 1/100,000 | 1404 |
| ,, ,, 1/1,000,000 | 1296 |
| Control normal serum 1/10 | 1600 |

Number of organisms implanted, 1328.

(*b*) Phagocytic experiment: 50 c.mm. of each mixture of blood and serum was added to 5 c.mm. of undiluted culture and incubated on the mixing machine for 15 minutes, when opsonic films were made (Table XI).

TABLE XI.

| Concentration of serum present in the blood. | Number of cocci in 100 polymorphs. |
|---|---|
| Patient's serum 1/10 | 1313 |
| ,, ,, 1/100 | 887 |
| ,, ,, 1/1000 | 686 |
| ,, ,, 1/10,000 | 593 |
| ,, ,, 1/100,000 | 546 |
| ,, ,, 1/1,000,000 | 557 |
| Control normal serum 1/10 | 277 |

From the relatively enormous dilution which the patient's serum may undergo before its effect ceases, it seems that the decrease in growth rate must be a direct consequence of the increased phagocytosis.

It has been shown in a previous section that heat-stable opsonins are responsible for the increased phagocytosis of virulent organisms which occurs when patients' serum is added to normal blood. The matter was further investigated, therefore, by an examination of the effect of heat on the property of the serum to affect the bactericidal power of the blood.

*Experiment 11.*

Portions of serum from a patient and a normal person were heated at 55° C. for 30 minutes. Phagocytic and bactericidal experiments were set up with the heated and unheated sera by the same methods as already described (Table XII).

TABLE XII.

| | *Growth experiment.* Survivors present after incubation for 1 hour in mixtures as indicated. | *Phagocytic experiment.* Number of microbes in 100 polymorphs. |
|---|---|---|
| Normal blood + 1/20 unheated patients' serum . | 272 | 997 |
| „ „ + 1/20 heated „ „ . | 352 | 939 |
| „ „ + 1/20 unheated normal „ . | 1976 | 362 |
| „ „ + 1/20 heated „ „ . | 2104 | 360 |

These experiments show that the increased phagocytosis of virulent streptococci which is a consequence of the addition of even minute quantities of the sera of septicæmia patients to normal blood is followed, or at least accompanied, by a considerable reduction in the rate of growth, or even actual destruction of the cocci in the blood. There can be little doubt that the growth-inhibiting effects conferred on normal blood by the serum of patients are due, in the main, to increased phagocytosis.

A further point of interest is that the opsonins in the sera which are responsible for this are heat-stable. The increased bactericidal power conferred on normal blood by the serum of patients is therefore due to heat-stable opsonins.

A similar phenomenon has been described by Robertson and his co-workers studying the sera of animals or human beings infected with pneumococci. It was found that after crisis in pneumonia the serum contained heat-stable opsonins, which even in high dilution rendered a mixture of cells and normal serum bactericidal for virulent pneumococci (Robertson, Woo, Cheers and King, 1928; and Sia, Robertson and Woo, 1928).

## DISCUSSION.

When the phagocytic power of normal human defibrinated blood was compared with that of patients infected with hæmolytic streptococci, it was found that although the leucocytes of the patient were, in reality, relatively inactive, their capacity to phagocyte virulent hæmolytic streptococci was much greater than that of the normal. This superiority proved to be due to the opsonic power of the serum, and was not demonstrable when the test was

conducted with avirulent microbes. These opsonins were found to be heat-stable and presumably to be identified with Neufeld's bacteriotropins.

Employing suitable methods the heat stability can be shown to be almost complete. In view of these results it is suggested that the partial heat stability of the immune opsonins in connection with other infections reported by Wright and others may find an explanation in the harmful effects of heated serum on the phagocytic capacity of the leucocytes employed in the test.

Little attention has been paid in the past to the significance of these heat-stable opsonins in human streptococcal infections. This may be due to the fact that most workers, following the method of Wright in employing emulsions made from agar cultures, were using microbes which are usually avirulent. The comparatively small variations in opsonic power in streptococcal infections recorded by Wright, Douglas, Freeman, Wells and Fleming (1907), Weinstein (1906), Schiffman and Kohn (1909), Potter (1907), Potter and Krumweide (1907), Ross (1907) and Tunnicliffe (1907) are presumably to be accounted for in this way.

It would thus seem advisable in future studies of the mechanism of immunity in streptococcal infections to employ virulent cultures.

The relation between phagocytic and bactericidal power of blood was next studied, and it has been shown that the addition of the serum of patients to normal blood leads to the phagocytosis and destruction of streptococci, which without this are resistant and able to multiply freely.

Analogous results have recently been reported by Robertson and his fellow workers in pneumococcus infections (1928). Sia, Robertson and Woo (1928) showed that the serum of cases of lobar pneumonia after crisis had a similar action in promoting the phagocytosis and destruction of virulent pneumococci by leucocytes. Armstrong (1925) had previously shown that such sera were able to protect mice against infection with virulent pneumococci. And it is highly probable that this protective action may be ascribed to the heat-stable opsonins.

There is no reason to suppose that the serum of cases infected with hæmolytic streptococci should act differently where streptococci are concerned. It has been shown that it assists bactericidal action even in high dilution *in vitro*, and probably the same holds *in vivo*. If this be the case it becomes necessary to inquire whether this property could be exploited for the treatment of established or imminent infections.

The patient with a fully established infection generally has a large supply of heat-stable opsonins in the serum. Injection of further quantities is probably redundant. But the recently infected case is in another category, and the early injection of such heat-stable opsonins might possibly be of value.

While a possible source of supply would be the serum of convalescents, the alternative is the serum of immunized animals. But ordinary antistreptococcal and antiscarlatinal sera apparently do not contain these heat-stable opsonins. I have tested a number of specimens. Neufeld and Rimpau (1905) it is true found them in antistreptococcal sera of their own manufacture and showed that such sera were able to protect animals, but most workers have apparently not tested for the presence of these bodies in their sera. It thus seems that some special conditions must be fulfilled for their production in animals.

Further work is obviously necessary to see how and in what way sera having these properties can be obtained, and whether they can be usefully employed in imminent infections. This has not yet been demonstrated.

---

I am very deeply indebted to Sir Almroth Wright and Dr. Leonard Colebrook for their interest and advice throughout the period of this research, and also to Dr. Morris of the Hammersmith Hospital, and Drs. Ruby Inkster and Breen of the Metropolitan Asylums Board, for taking specimens of blood.

## CONCLUSIONS.

1. The leucocytes in the defibrinated blood of patients infected with hæmolytic streptococci phagocyte virulent strains of hæmolytic streptococci in greater numbers than do the leucocytes of normal persons.

2. This is shown to be due for the most part to a high opsonic power in the serum.

3. This opsonic power is heat-stable, resisting 55°C. for 30 minutes.

4. When small quantities of such serum containing heat-stable opsonins are added to normal defibrinated blood, the rate of growth of virulent hæmolytic streptococci in that blood is materially reduced. This is shown to be due to increased phagocytosis of the microbes by the leucocytes.

## REFERENCES.

Armstrong, R. R.—(1925) *Proc. Roy. Soc.*, B, **98**, 525.
Colebrook, L.—(1925) *Proc. Roy. Soc. Med.* (joint discussion Sect. Med. Surg. Path.), **18**, 64.
Cummins, S. L., and Ackland, C. M.—(1924–25) *J. Hyg.*, **23**, 364.
Jotten, K. W.—(1917) *Arch. Gynæk.*, **107**, 59.
Neufeld, F., and Rimpau, W.—(1904) *Deuts. med. Wschr.*, **30**, 1458.—(1905) *Z. Hyg.*, **51**, 283.
Potter, N. B.—(1907) *J. Amer. Med. Assoc.*, **49**, 1815.
*Idem.*, and Krumwiede, C.—(1907) *J. Infect. Dis.*, **4**, 601.
Robertson, O. H., Woo, S. T., Cheers, S. N., and King, L. P.—(1928) *J. Exp. Med.*, **47**, 317.
Ross, G. W.—(1907) *J. Amer. Med. Assoc.*, **49**, 1245.
Schiffman, J., and Kohn, R.—(1909) *Wien. klin. Wschr.*, **22**, 82.
Shattock, S. G., and Dudgeon, L. S.—(1908) *Proc. Roy Soc.*, B, **80**, 165.
Sia, R. H., Robertson, O. H., and Woo, S. T.—(1928) *J. Exp. Med.*, **48**, 513.
Todd, E. W.—(1927[1]) *Brit. J. Exp. Path*, **8**, 1.—(1927[2]) *Ibid.*, **8**, 289.—(1927[3]) *Ibid.*, **8**, 361.
Tunnicliff, R.—(1907) *J. Infect. Dis.*, **4**, 304.
Weinstein, E. M.—(1906) *Berlin klin. Wschr.*, **43**, 1275.
Wright, Sir Almroth E., and Colebrook, L.—(1921) 'Technique of the Teat and Capillary Glass Tube,' London (Constable).
Wright, Sir Almroth E., Colebrook, L., and Storer, E. J.—(1923) *Lancet*, **1**, 305, 417, 475.
Wright, Sir Almroth E., and Reid, S. T.—(1906) *Proc. Roy. Soc.*, B, **77**, 194.
Wright, Sir Almroth E., Douglas, S. R., Freeman, J., Wells, J. H., and Fleming, A.—(1907) *Lancet*, **2**, 127.

# A COMPARISON OF THE PROPHYLACTIC VALUES OF THE H, O AND R ANTIGENS OF *SALMONELLA ÆRTRYCKE*, TOGETHER WITH SOME OBSERVATIONS ON THE TOXICITY OF ITS SMOOTH AND ROUGH VARIANTS.

HASSEIN MOHAMMED IBRAHAM (CAIRO),

AND

HARRY SCHÜTZE.

*From the Bacteriological Department, Lister Institute, London.*

Received for publication November 16th, 1928.

THE object of this research was to establish, if possible, the comparative prophylactic values of the various antigens that may be present in a bacterial vaccine.

The microbe employed was *S. ærtrycke* "Glasgow"; the antigens considered were the H and O of the smooth salt stable variant and the H and R of the rough salt sensitive variant, H being the thermolabile antigen associated with the flagellar substance and a flocculent agglutination precipitate, O and R being the thermostabile antigens associated with the somatic substance and a granular agglutination precipitate; the experimental animal was the mouse.

As a preliminary step, the toxicity of the strains was investigated in much the same way as, in this laboratory, Goyle (1926) investigated that of *S. enteritidis* and Cathcart (1906) both *S. enteritidis* and *S. paratyphosus B.*

The *ærtrycke* strain "Glasgow" was perfectly smooth, but by plating a several weeks old broth culture the rough variant was obtained. Both the rough and the smooth forms were shown by serological tests and the presence of motility to contain abundant H antigen.

## TOXICITY EXPERIMENTS.

Cultures of the two variants were grown on agar at 37° for 24 hours, washed down in saline, standardized by counting and subsequent dilution with saline, and killed by heating to the respective temperatures of 60° and 100° C.

Injections were either subcutaneous or intraperitoneal, as the experiment was planned. An observation term of 3 days was chosen as the most likely to elicit a toxic effect, and avoid the complication of infections secondary to an impairment of health.

*Experiments 1, 2, 3.*—Two types of bacterial emulsion, washed and unwashed, were used in these experiments. In the case of the former the saline emulsion before heating was shaken with beads for 1 hour, centrifuged, and the deposit resuspended in saline; this process was carried out six times in an endeavour to remove the outer flagellar portion of the bacterium and so possibly affect the

toxicity of the culture. After the last washing the emulsion was restandardized; it was then seen to how great an extent the bodies of the bacteria had by this process of washing been broken down and got rid of in the successive supernatants, for the original emulsions had a count of 160,000 millions, whereas the final suspensions represented in the case of the smooth variant 45,600 millions, in the case of the rough variant 57,600 millions.

One mouse was inoculated with each dilution; the inoculations were subcutaneous.

TABLE I.—S. ærtrycke "*Glasgow*" *Emulsions Inoculated Subcutaneously into Mice.*

Deaths within 3 days after inoculation with varying doses of bacteria. One mouse inoculated with each dilution. D = death; S = survival.

| Experiment. | Variant. | Emulsion. | $320\times10^9$ | $160\times10^9$ | $91\times10^9$ | $40\times10^9$ | $23\times10^9$ | $10\times10^9$ |
|---|---|---|---|---|---|---|---|---|
| 1 | Smooth | Unwashed, heated 60° 1½ hrs. | ... | D | ... | D | ... | S |
| 2 | „ | „ „ „ | ... | ... | D | D | S | S |
| 3 | „ | Washed, „ „ | ... | D | ... | D | ... | S |
| 1 | „ | Unwashed, heated 100° ½ hr. | ... | D | ... | S | ... | S |
| 3 | „ | Washed, „ „ | ... | D | ... | S | ... | S |
| 1 | Rough | Unwashed, heated 60° 1½ hrs. | D | D | ... | S | ... | S |
| 2 | „ | „ „ „ | D | D | ... | S | ... | ... |
| 3 | „ | Washed, „ „ | ... | S | ... | S | ... | S |
| 1 | „ | Unwashed, heated 100° ½ hr. | S | S | ... | S | ... | S |
| 3 | „ | Washed, „ „ | ... | S | ... | S | ... | S |

It is seen in the case of the smooth variant (*a*) that the repeated saline washings have not reduced the toxicity of the bacteria that remained unlysed by that process, (*b*) that heating for ½ hour at 100° has lowered the toxicity by about a quarter.

In the case of the rough variant, it is seen (*a*) that heated to 60° it has about a quarter of the toxicity of the smooth variant similarly treated, (*b*) that washing and heating to 100°, in this instance, both lower the toxicity. The reading of these results is, of course, purely tentative because of the small number of animals employed.

*Experiment 4.*—Unwashed emulsions of "Glasgow" were prepared as in Experiments 1, 2 and 3, but this time inoculation was intraperitoneal.

TABLE II.—S. ærtrycke "*Glasgow*" *Emulsions Inoculated Intraperitoneally into Mice.*

Deaths within 3 days after inoculation with varying doses of bacteria.
Numerator of fractions = number of deaths.
Denominator of fractions = number of mice inoculated.

| Variant. | Heated. | $96\times10^9$ | $48\times10^9$ | $24\times10^9$ | $12\times10^9$ | $6\times10^9$ | $3\times10^9$ | $1{\cdot}5\times10^9$ | $0{\cdot}75\times10^9$ | $0{\cdot}37\times10^9$ | $0{\cdot}18\times10^9$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Smooth | 60° 1½ hrs. | $\frac{1}{1}$ | $\frac{1}{1}$ | $\frac{1}{1}$ | $\frac{1}{1}$ | $\frac{1}{1}$ | $\frac{1}{1}$ | $\frac{5}{6}$ | $\frac{4}{10}$ | $\frac{3}{9}$ | $\frac{0}{6}$ |
| „ | 100° ½ hr. | ... | ... | ... | ... | $\frac{1}{1}$ | $\frac{2}{5}$ | $\frac{1}{5}$ | $\frac{0}{3}$ | ... | ... |
| Rough | 60° 1½ hrs. | ... | ... | $\frac{1}{1}$ | $\frac{4}{4}$ | $\frac{6}{9}$ | $\frac{1}{5}$ | $\frac{0}{4}$ | ... | ... | ... |
| „ | 100° ½ hr. | ... | $\frac{1}{1}$ | $\frac{1}{1}$ | $\frac{1}{1}$ | $\frac{2}{5}$ | $\frac{1}{6}$ | $\frac{0}{4}$ | ... | ... | ... |

Here again, as far as such small numbers afford an indication, and taking as index the amount needed to kill approximately 100% of the inoculated animals, the smooth variant heated to 60° is about four times as toxic as the smooth variant heated to 100°, or the rough variant whether heated to 60° or to 100°.

*Experiment 5.*—In this experiment filtered autolysates as employed by Cathcart and by Goyle were prepared. Saline emulsions of 24-hour agar culture of "Glasgow" were maintained at 37° for 8 days in the presence of toluol, filtered through Seitz filters and inoculated either unheated or heated to 100° for ½ hour. The dosage is expressed in terms of the original bacterial content of the emulsion and the inoculation was intraperitoneal.

TABLE III.—S. ærtrycke *"Glasgow" Autolysate Filtered and Inoculated Intraperitoneally into Mice.*

Deaths within 3 days after inoculation with varying doses of filtered autolysate.
Numerator of fractions = number of deaths.
Denominator of fractions = number of mice inoculated.

| Variant. | Heated. | $32\times10^9$ | $16\times10^9$ | $8\times10^9$ | $4\times10^9$ | $2\times10^9$ | $0{\cdot}5\times10^9$ | $0{\cdot}12\times10^9$ |
|---|---|---|---|---|---|---|---|---|
| Smooth | ... | ... | ... | $\frac{3}{3}$ | $\frac{1}{1}$ | $\frac{1}{4}$ | $\frac{0}{4}$ | $\frac{0}{1}$ |
| „ | 100° ½ hr. | ... | $\frac{1}{1}$ | $\frac{3}{4}$ | ... | $\frac{1}{4}$ | $\frac{1}{4}$ | ... |
| Rough | ... | $\frac{1}{2}$ | $\frac{0}{1}$ | $\frac{0}{2}$ | ... | $\frac{0}{1}$ | $\frac{0}{1}$ | ... |
| „ | 100° ½ hr. | $\frac{0}{2}$ | $\frac{0}{1}$ | $\frac{0}{3}$ | ... | $\frac{0}{1}$ | $\frac{0}{1}$ | ... |

The toxicity of these filtered autolysates is, as might be expected, less than that of the corresponding whole emulsions as demonstrated in Table II. To kill approximately 100% of the inoculated mice, using the smooth variant and not heating to 100°, a dose of filtrate corresponding to 4000 millions is needed, whereas unautolysed bacteria will, under similar conditions, do the same in a dosage of something like 1500 millions.

In Experiment 5, as in the previous ones, heating to 100° lowers the toxicity of the smooth variant to a quarter; the rough variant, whether heated or not, is considerably less toxic; possibly lysis does not take place so actively in this variant as it does in the case of the smooth.

Attempts to demonstrate an exotoxin in the filtrates of one-day broth cultures and to compare them as regards their heat stability and their dependence on the smoothness or roughness of the parent strain failed. As much as 3 c.c.—already an impossible inoculation for a mouse—did not kill. Even evaporation at a low temperature of such filtrates did not help; a quantity corresponding to 4 c.c. of the original filtrate was not lethal, and this approaches the amount of uninoculated broth which, given in similar concentrated form, will itself cause the death of a mouse.

## IMMUNITY EXPERIMENTS.

Working with two different species of bacteria and employing different kinds of experimental animals, Arkwright (1927) and Springut (1927) have arrived at divergent conclusions with regard to the immunizing properties of the H and O antigens of their vaccines.

Arkwright's conclusion that the thermostabile O antigen was alone responsible for the production of immunity was founded on his observation of the effect of *B. typhosus* and *B. paratyphosus A* inoculations in the guinea-pig. Springut's contradictory opinion that it was the thermolabile H antigen which constituted the effective portion of a vaccine was based on experiments with mice inoculated with *B. typhi murium*—an organism which may have been *S. ærtrycke.*

In the experiments to be described here the two variants of "Glasgow" already used in the toxicity experiments were again taken and from them a series of five different vaccines was prepared.

Agar cultures grown at 37° for 24 hours were emulsified in saline and standardized by counting and diluting with saline; after division into halves the vaccines were killed by heating for 1½ hours at 60° and 2 hours at 100° respectively. In this way four vaccines were obtained:

1. "Smooth variant" vaccine, heated to 60° for 1½ hours, containing both H and O antigens.
2. "Smooth variant" vaccine, heated to 100° for 2 hours, containing only O antigen.
3. "Rough variant" vaccine, heated to 60° for 1½ hours, containing both H and R antigens.
4. "Rough variant" vaccine, heated to 100° for 2 hours, containing only R antigen.

In addition, a second O vaccine (2*a*) was prepared in the manner described by Springut, viz. by growing the smooth variant on a 0·15% phenol agar, which largely inhibits the formation of H antigen, and heating the vaccine to 100° for 1 hour.

These five vaccines were now tested for the accuracy of their supposed antigenic content by inoculation into rabbits and titration of the agglutinins of their sera. The rabbits were inoculated intravenously with 500 million, 2000 million and 8000 million doses at intervals of a week. The following table gives the titres that were obtained:

TABLE IV.

| Rabbit sera. | Antigen theoretically present. | Titre with agglutinating emulsions. | | | |
|---|---|---|---|---|---|
| | | Smooth, unheated. | Smooth, heated 100° 1 hr. | Rough, unheated. | Rough, heated 100° 1 hr. |
| 1 | H + O | 1/12800 | 1/12800 | ... | ... |
| 2 | O | 1/3200 | 1/3200 | ... | ... |
| 2*a* | O | 1/1600 | 1/1600 | ... | ... |
| 3 | H + R | 1/3200 | < 1/100 | 1/6400 | 1/400 |
| 4 | R | < 1/100 | < 1/100 | 1/400 | 1/400 |

In Table IV from the agglutinations with heated culture one can deduce the presence of effective O antigen in vaccines 1, 2 and 2*a*, and of effective R antigen in vaccines 3 and 4, for the corresponding agglutinins are present in these rabbit sera.

Agglutination with smooth unheated culture proves that vaccine 3 possesses H antigen but that vaccine 4 does not. In order to demonstrate clearly the

presence of H antigen in vaccine 1 and to make certain that 2 and 2*a* contained none, it was necessary to absorb the O agglutinins in their corresponding sera and examine for any residual H agglutinins.

Rabbits 2 and 2*a* received a further inoculation of 8000 million organisms so that there might be a still greater chance of detecting even a slight trace of H antigen in the vaccine, and the resultant sera together with serum 1 were absorbed with smooth cultures heated to 100° for 2 hours and smooth culture unheated and heated to 100° used for agglutination. In this way all the O agglutinins were absorbed and any H agglutinins that might be present could be revealed.

TABLE V.

| Sera absorbed with smooth variant heated 100° 2 hrs. | Titre with agglutinating emulsions. | |
|---|---|---|
| | Smooth, unheated. | Smooth, heated 100° 1 hr. |
| 2 | $\frac{1}{200}$ | $< \frac{1}{100}$ |
| 2*a* | $\frac{1}{800}$ | $< \frac{1}{100}$ |
| 1 | $\frac{1}{64000}$ | $< \frac{1}{100}$ |

Table V shows that sera 2 and 2*a* possess low H titres; one may conclude, therefore, that in spite of heating to 100° a very small amount of H antigen has remained agglutinogenically effective in these O vaccines. This is more particularly the case with the phenol agar grown vaccine 2*a*, the reason no doubt being the shorter exposure to a temperature of 100°. Vaccine 1 has stimulated the production of a good H titre.

Therefore, apart from this incomplete destruction of H antigen in the O vaccines 2 and 2*a*, inoculation into rabbits has demonstrated that the various intended types of vaccine have been achieved.

## IMMUNIZATION OF EXPERIMENTAL ANIMALS.

Immunization of mice was now carried out by the subcutaneous inoculation of these vaccines in $\frac{1}{2}$ c.c. amounts.

The animals were housed separately in glass jars, batches of 15 being treated with each type of vaccine. The doses were 200 millions, followed after 7 days by 800 millions, and 14 days after the second dose by 3000 millions. Fourteen days later the test-dose of $\frac{1}{2}$ c.c. of a 1 in 20 broth dilution of a 24-hour broth culture of "Glasgow" (smooth variant) was injected intraperitoneally. This route was chosen in preference to the oral one because of its accuracy and simplicity, Webster (1922) having shown that subcutaneous immunization affords protection against either method of infection, though the intraperitoneal test is the more severe.

Fifteen normal mice received simultaneously the same test-dose, and diminishing amounts were given to further mice so that some idea of the number of m.l.d. used might be obtained.

The test-dose represented some 500 m.l.d. Weight curves showed that the animals withstood the inoculations well; indeed, of the 75 mice that were given vaccine, only two died during immunization, one after the first dose of

TABLE VI.—*Mice Immunized Subcutaneously with $200 \times 10^6$, $800 \times 10^6$ and $3000 \times 10^6$ S. ærtrycke with Intervals of 7 and 14 Days.*

Test dose = ½ c.c. of $\frac{1}{20}$ dilution of 24-hour broth culture of *S. ærtrycke* (smooth variant) injected intraperitoneally 14 days after last immunizing dose.

| Vaccine. | | | | | | Number of mice dead in days after test dose. | | | | | | | | | | | | Percentage of survivors after 80 days. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Variant. | Heated. Hours. | Heated. Temp. | Antigen present. | Number of mice used. | 1. | 2. | 3. | 4. | 5. | 6. | 7. | 10. | 14. | 32. | 50. | 80. | |
| 1 | Smooth | 1½ | 60° | H + O | 14 | ... | ... | ... | ... | ... | . | ... | ... | 1 | 5 | 5 | 7 | 50 |
| 2 | „ | 2 | 100° | O* | 15 | 1 | 3 | 3 | 4 | 4 | 5 | 5 | 9 | 10 | 12 | 12 | 12 | 20 |
| 2*a* | „ (phenol agar grown) | 1 | 100° | O* | 15 | ... | 2 | 2 | 2 | 2 | 2 | 2 | 5 | 6 | 10 | 10 | 10 | 33 |
| 3 | Rough | 1½ | 60° | H + R | 15 | 1 | 6 | 6 | 6 | 8 | 8 | 11 | 13 | 14 | 14 | 14 | 14 | 7 |
| 4 | „ | 2 | 100° | R | 14 | ... | 7 | 7 | 9 | 10 | 11 | 12 | 14 | 14 | 14 | 14 | 14 | 0 |
| * Some H antigen present. | | | | | | | | | | | | | | | | | | |
| Control mice, full test dose | | | | | 15 | 9 | 12 | 12 | 12 | 14 | 14 | 14 | 15 | 15 | 15 | 15 | 15 | 0 |
| „ | $\frac{1}{5}$ „ | | | | 2 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| „ | $\frac{1}{50}$ „ | | | | 2 | ... | ... | ... | ... | ... | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 0 |
| „ | $\frac{1}{500}$ „ | | | | 2 | ... | ... | ... | ... | ... | ... | ... | 1 | 1 | 2 | 2 | 2 | 0 |

*S. ærtrycke* was recovered from the heart-blood of all the mice that died, with the exception of one mouse in Vaccine 1 group, which died on the 53rd day; here the heart-blood specimen was sterile.

vaccine 4 and one after the second dose of vaccine 1, a coliform bacillus being isolated from the heart-blood of each case. Heart-blood cultures were made from each animal that died.

From Table VI one can conclude that the vaccine to give the most definite and prolonged protection was vaccine 1, which contained both H and O antigen. Ten days after infection, when the last belated death among the control mice took place (the great majority of deaths having occurred within 2 days), there were no deaths to record among the mice immunized with this H + O vaccine; 80 days after infection 50% were still alive.

Vaccine prepared from the rough variant, whether it contained H or not, was ineffective.

The O vaccines, which, as we have seen, contained traces of H antigen, have afforded protection, apparently in proportion to the amount of H antigen that had survived the steaming, but it would be erroneous to suppose that this slight immunity had been occasioned by the H antigen itself, because vaccine 3, which contained abundant H antigen, failed to do more than slightly retard the date of death.

Although the O vaccines do not prevent the occurrence of late deaths, the significance of which must remain a matter of speculation until more data are available, they do occasion a very definite delay in the death-rate, so that at a time when all the control mice are dead 50% of those immunized with O vaccine are still alive.

One must, therefore, assume either that protection is afforded by the simultaneous action of both H and O antigens, or that in the process of destroying H antigens the immunogenic properties of the O vaccine likewise deteriorate. Further experiments will be needed to elucidate this point.

## DISCUSSION.

The results obtained here with *S. ærtrycke* may be taken as being in agreement with those of Springut, though the theory he puts forward to explain his facts will not fit the case as established by us.

Springut, finding steamed smooth vaccine deficient both in H antigen and immunogenic power, came to the conclusion that his vaccine depended on the former for the possession of the latter. By the use of the rough variant we were able to show that H antigen by itself has no immunizing influence.

A comparison with the results obtained by Arkwright is rendered difficult by the fact that he worked with a different experimental animal and bacteria of considerably different biological properties. The fact that steaming his *B. typhosus* and *B. paratyphosus A* vaccines did not damage their immunogenic properties does not tally with our findings; how far that discrepancy may be explained by the presence of a heat-labile toxin in *S. ærtrycke* and the absence of it in *B. typhosus* and *B. paratyphosus A* remains to be seen; it is possible that immunity to infection with *S. ærtrycke* is based on the possession by the animal of antitoxic as well as bactericidal bodies, and this may not be the case with the two other organisms mentioned. That H antigen itself, as exemplified by a rough variant vaccine containing H and R antigens, does not protect, is established by the results of Arkwright's work as well as ours.

SUMMARY.

1. The rough variant of *S. ærtrycke* "Glasgow" possesses about a quarter of the toxicity of its smooth counterpart.

2. Heating to 100° lowers the toxicity of the smooth variant by about a quarter, but the toxins of the rough variant do not appear to be so labile.

These results agree in principle with those published by Goyle and with those of Cathcart in respect of the smooth variant.

3. Repeated washings with saline, undertaken with the view to removing the outer flagellar portion of the bacterium, do not lower the toxicity of the smooth variant.

4. Three agglutinogenic antigens (H, O and R) have been considered in relationship to the immunity conferred on mice by inoculation with *S. ærtrycke* vaccine.

5. Only vaccine containing agglutinogenically active H and O antigens in combination has been shown to be effective, 50% of the animals inoculated surviving for 80 days a test-dose of 500 m.l.d.

6. Vaccines containing only O antigen did not give prolonged protection, though they delayed death very definitely.

7. Vaccines containing only R or H + R antigens may be regarded as ineffective. That is to say, a vaccine not heated above 60° and deriving from a smooth variant culture of *S. ærtrycke* is capable of conferring good and lasting immunity against infection with the living homologous strain, whereas a similar vaccine heated to 100° for the most part merely delays death, while a vaccine deriving from a rough variant culture of the same microbe, whether heated to 100° or merely to 60°, is incapable of immunizing. This failure may be due to the lack of combined H and O antigen in these O, R and H + R vaccines, or the fact that heating smooth variant culture to 100° in order to destroy the thermolabile H antigen coincidentally damages it as an immunizing agent.

## REFERENCES.

ARKWRIGHT, J. A.—(1927) *J. Path. Bact.*, **30**, 345.
CATHCART, E. P.—(1906) *J. Hyg.*, **6**, 112.
GOYLE, A. N.—(1926) *J. Path. Bact.*, **29**, 365.
SPRINGUT, E.—(1927) *Z. ImmunForsch.*, Orig., **52**, 25.
WEBSTER, L. T.—(1922) *J. Exp. Med.*, **36**, 71.

# INDEX TO AUTHORS.


ALSTON, J. N., a study of the bactericidal power of whole blood by the method of culture in slide-cells, 300
ANDERSON, W., see *Magee, H. E.*

BEDSON, S. P., observations on the mode of action of a viricidal serum, 235
——— and BLAND, J. O. W., on the supposed relationship between the viruses of herpes febrilis and vaccinia, 174
BLAND, J. O. W., the suitability of the guinea-pig for experimental work with vaccinia virus, 97
——— filter and centrifuge experiments with guinea-pig vaccinia virus, 283
——— see *Bedson, S. P.*
BULLOCH, W., see *Candy, H.*
BUTTLE, G. A. H., and TREVAN, J. W., the action of *Vibrion septique* and *B. welchii* toxin on isolated organs, 182
BYROM, F. B., and KAY, H. D., blood phosphorus in health and disease: III.—A. The alleged value of blood phosphorus determinations in suspected malignant disease. B. The phosphorus distribution in anæmia, polycythæmia and leukæmia, 72

CANDY, H., and BULLOCH, W., experiments and observations on the contact of animal tissue with biniodide of mercury dissolved in aqueous potassium iodide, 179
COWELL, S. J., the influence of dietetic factors on the intensity of symptoms produced during acute experimental nephritis, 164
CRAIGIE, J., a method of staining bacterial flagella, 55
CRUICKSHANK, J., see *Downie, A. W.*
CRUICKSHANK, R., the experimental transformation of the intestinal flora, 318

DALLING, T., GLENNY, A. T., MASON, J. H., and O'BRIEN, R. A., the testing and standardization of *B. welchii* (*perfringens*) antitoxin, 43
DAY, H. B., the preparation of antigenic specific substance from *Staphylococcus pyogenes aureus*, 198
DOUGLAS, S. R., and SMITH, W., cataphoresis experiments with the virus of vaccinia, 213
DOWNIE, A. W., and CRUICKSHANK, J., the resistance of *Streptococcus fæcalis* to acid and alkaline media, 171
DRUMMOND, J. C., see *Parkes, A. S.*
DUDLEY, S. F., natural and artificial stimuli in the production of human diphtheria antitoxin, 290

EAGLES, G. H., colony variants in hæmolytic streptococci, 330
EIDINOW, A., see *Galloway, I. A.*

FAIRBROTHER, R. W., the structure of the *V. choleræ*, with reference to its immunizing properties, 89
FELIX, A., and ROBERTSON, M., serological studies in the group of the spore-bearing anaerobes. I. The qualitative analysis of the bacterial antigens of *B. œdematis maligni* (*Vibrion septique*) and *B. tetani*, 6
FINDLAY, G. M., a note on the cultivation of the virus of fowl-pox, 28
FLEMING, A., on the influence of temperature on the rate of agglutination of bacteria, 231
FOWWEATHER, F. S., some notes on the preparation of gold sols for the Lange test, with an account of a new method, 161

GALLOWAY, I. A., and EIDINOW, A., effect of ultraviolet light on the viability of the virus of foot and mouth disease, 326
GARROD, L. P., filter-passing anaerobes in the upper respiratory tract, 155
GLENNIE, A. E., see *Magee, H. E.*
GLENNY, A. T., see *Dalling, T.*

HARE, R., on the phagocytosis of hæmolytic streptococci of high and low virulence by the blood of patients infected with that organism, 337
HARTLEY, P., experiments on the purification and concentration of scarlet fever toxin, 259
HERBERT, F. K., pancreatic fat necrosis: a chemical study, 57
HILL, L., the capillary blood-pressure in relation to œdema and cerebral and intra-ocular pressure, 135
——— and MCQUEEN, J. M., the principle of capillary blood-pressure measurements as applied to glomerular and tubular function in the kidney, 127

IBRAHAM, H. N., and SCHÜTZE, H., a comparison of the prophylactic values of the H, O and R antigens of *Salmonella aertrycke*, together with some observations on the toxicity of its smooth and rough variants, 353

KERRIN, J. C., the incidence of *B. tetani* in human fæces, 69

LEDINGHAM, J. C. G., and MCCLEAN, D., the propagation of vaccine virus in the rabbit dermis, 216

MCCLEAN, D., see *Ledingham, J. C. G.*
MCQUEEN, J. M., see *Hill, L.*
MAGEE, H. E., ANDERSON, W., and GLENNIE, A. E., some observations on the effects of ether anæsthesia on the composition of the blood and their relation to thyroid function, 119
MASON, J. H., see *Dalling, T.*
MOTTRAM, J. C., the effect of radiation on Rous chicken tumour, 147
——— on the division of cells under varying tensions of carbon dioxide, 240

NEWMAN, C. E., bilirubin and the van den Bergh reaction, 112

O'BRIEN, R. A. see *Dalling, T.*
OKELL, C. C., and PARISH, H. J., vaccination of guinea-pigs against tuberculosis with B.C.G., 34

PARISH, H. J., see *Okell, C. C.*
PARKER, H. B., rough and smooth strains of *Corynebacterium diphtheriæ* on trypsin-serum agar, 207
PARKES, A. S., and DRUMMOND, J. C., the relation between fatness and sterility, 63
POPE, C. G., a simple electrometric comparator for the determination of hydrogen ion concentration, 225
PULVERTAFT, R. J. V., on the concentration and purification of streptococcal toxin, 276

RIDLEY, F., an apparatus for mixing cultures or other fluids, 253
ROBERTS, W. M., observations on the nature of the van den Bergh reaction, 107
ROBERTSON, M., see *Felix, A.*
ROBINSON, S. H. C., comparison between the antigenic properties of whole and lipoid-free sera, 299
RUCKER, E. A., and WHITBY, L. E. H., serodiagnosis of syphilis by Meinicke's macroreaction and microreaction, 30

SCHÜTZE, H., see *Ibraham, H. N.*
SMIRK, F. H.: I. The accurate measurement of the proportion of corpuscles and serum in blood. II. The volume of red blood-corpuscles in venous and oxygenated blood and after exposure to various saturations of carbon dioxide, 81
SMITH, J., the modification of scarlatinal toxin by formaldehyde, 49
SMITH, W., see *Douglas, S. R.*

TODD, C., experiments on the virus of fowl plague (I), 19
——— ——— (II), 101
——— on a dilution phenomenon observed in the titration of the serum of fowls immunized against the virus of fowl plague, 244
TODD, E. W., further observations on the virulence of hæmolytic streptococci, with special reference to the morphology of the colonies, 1
TREVAN, J. W., see *Buttle, G. A. H.*

WHITBY, L. E. H., see *Rucker, E. A.*

JAN 10 1929

VOL. IX. No. 6] [DECEMBER, 1928.

Medical Lib.

# THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

*Editors:*

E. C. DODDS
J. C. DRUMMOND
C. L. EVANS
P. FILDES
H. W. FLOREY
W. E. GYE
H. D. KAY
E. H. KETTLE
J. McINTOSH
J. W. McLEOD
J. A. MURRAY
W. J. TULLOCH

*With the Support of:*

F. W. ANDREWES (London)
A. E. BOYCOTT (London)
W. BULLOCH (London)
S. L. CUMMINS (Cardiff)
H. H. DALE (London)
H. R. DEAN (Cambridge)
A. M. DRENNAN (Otago, N.Z.)
G. DREYER (Oxford)
J. W. H. EYRE (London)
LEONARD FINDLAY (Glasgow)
DUNCAN GRAHAM (Toronto)
R. T. HEWLETT (London)
F. G. HOPKINS (Cambridge)
C. H. KELLAWAY (Melbourne)
J. C. G. LEDINGHAM (London)
T. J. MACKIE (Edinburgh)
H. OERTEL (Montreal)
LEONARD ROGERS (London)
C. S. SHERRINGTON (Oxford)
A. E. WRIGHT (London)

---

LONDON: H. K. LEWIS & CO. LTD., 136, GOWER STREET, W.C.1.

EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C.1.

CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

*Annual Subscription, January to December, post free, Two Pounds net.*
*Single Numbers, Seven and Sixpence net.*

# THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

## EDITORIAL NOTICE TO CONTRIBUTORS.

**Scope of Papers.**—This Journal was founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, pharmacological, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects. The Editors request all contributors to confine their papers chiefly to a description of their experimental work, and to reduce to a minimum reviews of previous work and theoretical discussions of their results.

**Forwarding Papers.**—Papers should be addressed to—

THE EDITORS,
*British Journal of Experimental Pathology,*
28, GOWER PLACE,
LONDON, W.C. 1,

or to any of the Editors personally.

**Date of Publication.**—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

**Rapidity of Publication.**—All papers accepted for publication will be printed, so far as possible, in the next following issue of the Journal, if received before the middle of the month preceding publication. Long papers, or papers with illustrations, may, however, be delayed if not received earlier.

**Conditions of Publication.**—Contributors should prepare their manuscripts with the greatest attention to detail. The fact of submitting a paper for publication will be taken by the Editors as an admission by the Author of his liability to pay for all unreasonable alterations made by him in proofs, and for mistakes due to lack of clearness in the preparation of the manuscript.

**References.**—Authors are requested to prepare their references in strict accordance with the method adopted in current issues of this Journal.

**Proofs.**—To avoid delay, proofs will not be submitted to Authors overseas unless they express a desire to see them, or unless the Editors think it necessary. Overseas Authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that error need not arise.

**Reprints.**—25 reprints will be supplied free to authors. Additional copies must be ordered from the Publishers *before the issue is printed,* and will be charged for according to the following scale:

*Number of Additional Reprints with Covers.*

| *Number of pages.* | 25 | 50 | 75 | 100 | 150 | 200 |
|---|---|---|---|---|---|---|
| | £ s. d. | £ s. d. | £ s. d. | £ s. d. | £ s. d. | £ s. d. |
| 1–4 | 1 4 6 | 1 6 0 | 1 7 6 | 1 8 6 | 1 12 6 | 1 15 0 |
| 5–8 | 1 11 6 | 1 13 6 | 1 15 6 | 1 17 6 | 2 3 0 | 2 6 6 |
| 9–12 | 2 1 0 | 2 4 0 | 2 7 0 | 2 10 0 | 2 15 6 | 3 0 0 |
| 13–16 | 2 8 0 | 2 11 6 | 2 15 0 | 2 18 0 | 3 5 0 | 3 11 0 |

*Special quotations may be obtained for Reprints without Covers.*

*Made and printed in Great Britain.*

BAIRD & TATLOCK (LONDON) LTD

An Improved Form of

# ANAËROBIC JAR

By Drs. McINTOSH & FILDES.

(*Reference: 'British Journal of Experimental Pathology,' 1921, II, 153.*)

B. 1488. **Price £3 15s. each**

Sole Address:

14-15 CROSS STREET HATTON GARDEN LONDON EC1

*Subscription in India and abroad Rs.* **16** *per annum. Post free. Payable in advance.*

— THE —

# INDIAN JOURNAL OF MEDICAL RESEARCH

**Edited by THE DIRECTOR-GENERAL, INDIAN MEDICAL SERVICE, THE PUBLIC HEALTH COMMISSIONER WITH THE GOVT. OF INDIA and THE DIRECTOR OF MEDICAL RESEARCH in collaboration with the leading authorities on Tropical Medicine.**

**The Indian Journal of Medical Research is published quarterly. Each number contains about 200 pages and is Illustrated with Plates in Colour and Half-tone and with Diagrams and Charts, and is the only medium publishing the results of the best medical research work being carried on by leading scientific medical men in India and the East.**

**THACKER, SPINK & CO., P.O. Box 54, CALCUTTA.**

**London Agents: W. THACKER & Co., 2, Creed Lane, E.C. 4.**

## RATES FOR ADVERTISEMENTS.

| | One insertion | Six (each) | Twelve (each) |
|---|---|---|---|
| | £ s. d. | £ s. d. | £ s. d. |
| Whole page ... ... ... ... | 4 10 0 | 4 5 0 | 4 0 0 |
| Half page ... ... ... ... | 2 10 0 | 2 5 0 | 2 2 6 |
| Quarter page ... ... ... ... | 1 7 6 | 1 5 0 | 1 2 6 |

All communications respecting Advertisements in this Journal should be addressed to the ADVERTISEMENT MANAGER, 27, Southampton Street, London, W.C. 2. Telephone: Gerrard 0693.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

VOL. IX, NO. 6. DECEMBER, 1928.

## CONTENTS.

BLAND, J. O. W.—FILTER AND CENTRIFUGE EXPERIMENTS WITH GUINEA-PIG VACCINIA VIRUS . . . . . 283

DUDLEY, SHELDON F.—NATURAL AND ARTIFICIAL STIMULI IN THE PRODUCTION OF HUMAN DIPHTHERIA ANTITOXIN . . . . . 290

ROBINSON, SYBIL H. C.—A COMPARISON BETWEEN THE ANTIGENIC PROPERTIES OF WHOLE AND LIPOID-FREE SERA . . . . . 299

ALSTON, J. M.—A STUDY OF THE BACTERICIDAL POWER OF "WHOLE" BLOOD BY THE METHOD OF CULTURE IN SLIDE-CELLS . . . . . 300

CRUICKSHANK, ROBERT.—THE EXPERIMENTAL TRANSFORMATION OF THE INTESTINAL FLORA . . . . . 318

GALLOWAY, I. A., AND EIDINOW, A.—EFFECT OF ULTRA-VIOLET LIGHT ON THE VIABILITY OF THE VIRUS OF FOOT AND MOUTH DISEASE . . . . 326

EAGLES, G. HARDY.—COLONY VARIANTS IN HÆMOLYTIC STREPTOCOCCI . . 330

HARE, RONALD.—ON THE PHAGOCYTOSIS OF HÆMOLYTIC STREPTOCOCCI OF HIGH AND LOW VIRULENCE BY THE BLOOD OF PATIENTS INFECTED WITH THAT ORGANISM . . . . . 337

IBRAHAM, HASSEIN MOHAMMED, AND SCHÜTZE, HARRY.—A COMPARISON OF THE PROPHYLACTIC VALUES OF THE H, O AND R ANTIGENS OF *SALMONELLA ÆRTRYCKE*, TOGETHER WITH SOME OBSERVATIONS ON THE TOXICITY OF ITS SMOOTH AND ROUGH VARIANTS . . . . . 353

TITLE-PAGE AND INDEX TO VOL. IX.

Subscriptions and Business Correspondence should be addressed to the Publishers, 28, Gower Place, London, W.C. 1. The JOURNAL is only sold *at the subscription rate* by the year or volume. General correspondence for the Editors should be addressed to Dr. P. FILDES, London Hospital, E. 1, but letters relating to biochemical and physiological papers to Professor E. C. DODDS, Middlesex Hospital, W. 1.

PRINTED BY ADLARD AND SON, LIMITED, LONDON AND DORKING.